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INTERNAL SECRETORY CAPACITY OF THE ABDOMINAL TESTIS IN THE GUINEA PIG[1]

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CLARIFICATION of the problem of the internal secretory capacity of the abdominal testis has never been achieved. Clinical reports indicate that some patients have a normal masculinity whereas others are eunuchoid (1, 2, 3, 4). Engberg (5) concluded from assays of urinary androgens from bilaterally cryptorchid men that retained testes produce less androgenic hormone than normal testes. Data from laboratory and other domesticated species are equivocal. Extracts of testes from cryptorchid domestic pigs contained approximately one-half as much hormone per unit weight as did extracts from scrotal testes (6). The level of 17-ketosteroid excretion by cryptorchid rabbits four weeks following surgery was essentially the same as that by castrates (7). Subsequent castration of the cryptorchids produced no alteration in the quantity secreted. Nelson (8) found evidence from the appearance of the seminal vesicles, prostates, and pituitaries that the production of male hormone by the rat was reduced by a very low level after 8 months of cryptorchidism. Moore and Gallagher (9), on the other hand, had concluded that hormone secretion by the guinea pig testis was not modified after 8 months of abdominal confinement, and Moore (10) could find no indication of a progressive decrease in the hormone secretion in rats cryptorchid for 314 to 392 days.

A re-investigation of the problem has been undertaken, using guinea pigs made cryptorchid within 2 days after birth and followed at intervals for 3 years. Sexual behavior and genital tract tissues were the indicators of testis hormone function.

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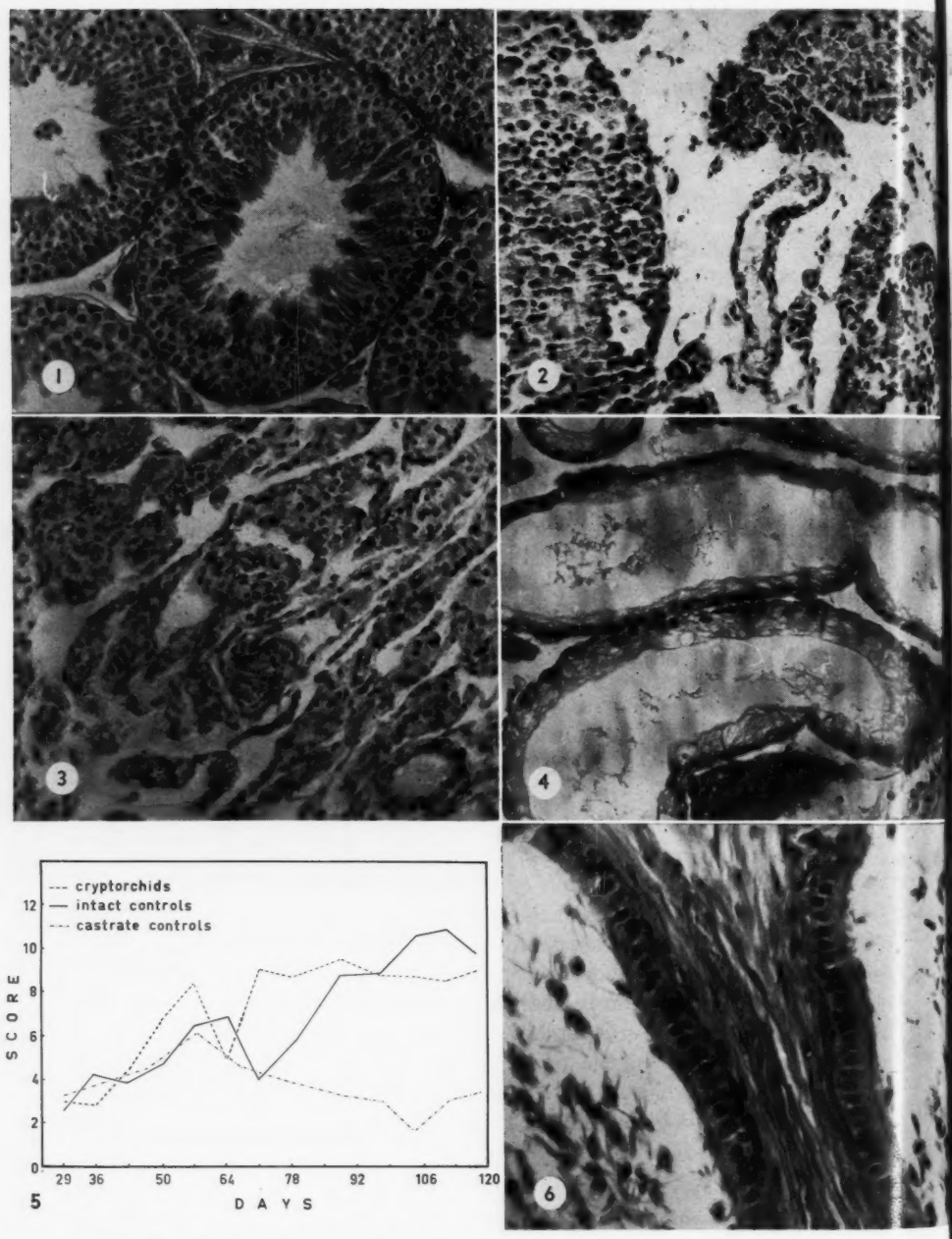

FIG. 1. Testis from an intact guinea pig. ×350.

FIG. 2. Testis from a cryptorchid guinea pig showing extreme atrophy. Tubules

MATERIAL AND METHODS

Twelve male guinea pigs were selected from a strain in which the males are characteristically vigorous in their sexual behavior. One to 2 days after birth 5 were made cryptorchid and 2 were castrated. An inch-long incision was made in the abdomen just lateral to the midline, extending posterior from slightly above the umbilicus. Each testis was drawn through the inguinal canal into the abdomen. The gubernaculum was severed. The testis was not confined in any way. Castration was performed via the scrotum. The remaining 5 animals served as the intact controls. All the animals remained with their mothers until day 17 when they were weaned. They were then caged alone in single cages for 3 years.

The first observations of sexual behavior were begun between days 25 and 29 and were continued at weekly intervals until day 120. The interval from birth to day 85 was considered to be the period of prepuberal development. The interval from day 85 to day 120 was considered postpubertal. Following these tests, the animals were not tested for 180 days. Five tests of sexual behavior were then given during each of the intervals between days 300 and 335, 730 and 765, and 1095 and 1130.

Throughout the period of examination the temperature of the laboratory was maintained at 70° to 75° F. The diet consisted of commercial rabbit pellets, oats, alfalfa hay, green vegetables, and water.

The method of testing and estimating the level of sexual behavior was in accordance with that described by Young and Grunt (12) and by Valenstein, Riss and Young (13). After the last series of tests, the animals were weighed and killed by exsanguination. The following tissues were removed, weighed when possible, fixed, and prepared for histological examination: testes, epididymides, seminal vesicles, dorsal prostate, pituitary, and penis. The pituitary was fixed in Zenker-formol fluid and stained by the Mallory-azan procedure. The remaining tissues were fixed in Bouin's fluid and stained with the Groat tetrachrome stain (14).

RESULTS

Examination of the testes at the time of autopsy, and later microscopically, provided abundant evidence that all had been confined in the abdomen. They were small, barely distinguishable masses embedded in the adipose tissue capping the anterior pole and extending laterally along the caput and corpus epididymidis. The microscopic appearance was identical with that of the abdominal testes described by Moore (15), Cooper (16), Rea (2) and Nelson (17). Different degrees of atrophy were apparent (Figs. 1, 2, 3, 4) and were probably correlated with the position after the operation. With the end points used, no evidence of a correlation was found between the degree of atrophy of the testes and their endocrine activity.

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interspersed with interstitial cells in lower right, blood vessels in center, interstitial cell masses elsewhere. ×350.

FIG. 3. Testis from a cryptorchid guinea pig. The condition is intermediate between that shown in Figs. 2 and 4. ×350.

FIG. 4. Testis from a cryptorchid guinea pig showing less atrophy. ×350.

FIG. 5. Maturation of sexual behavior in cryptorchid guinea pigs.

FIG. 6. Epididymis from an intact guinea pig. ×350.

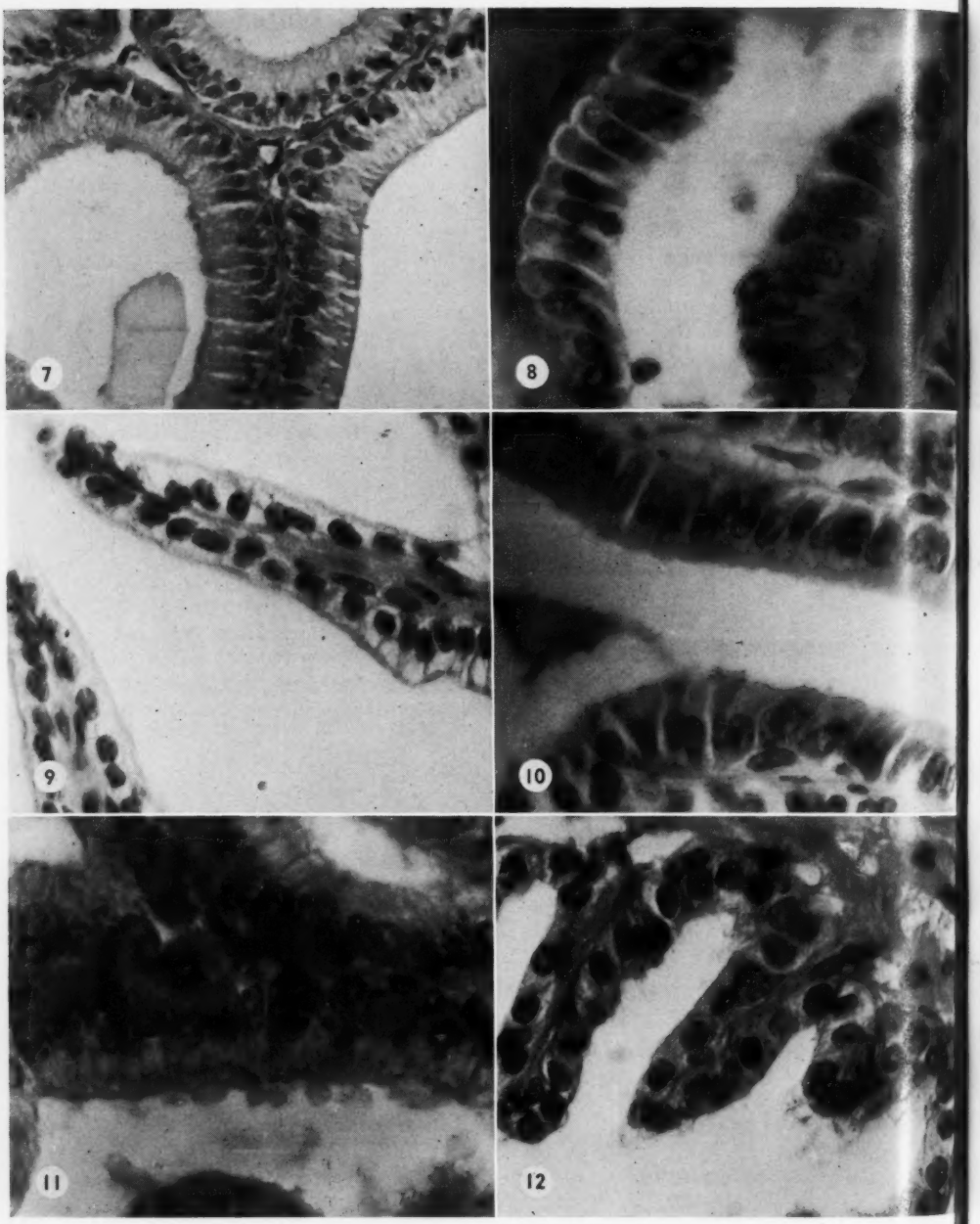

FIG. 7. Epididymis from the cryptorchid guinea pig testis in Fig. 3. ×350.
FIG. 8. Seminal vesicle epithelium from an intact guinea pig. ×1000.
FIG. 9. Seminal vesicle epithelium from a castrate guinea pig. ×1000.
FIG. 10. Seminal vesicle epithelium from a cryptorchid guinea pig. ×1000.
FIG. 11. Prostate epithelium from an intact guinea pig. ×1000.
FIG. 12. Prostate epithelium from a castrate guinea pig. ×1000.

At no time during the 3 years of the experiment did the sexual behavior scores of the cryptorchid and the control animals differ significantly. Development of sexual behavior during the prepuberal period was not different in the two groups of males (Fig. 5). The average age at the first ejaculation was about the same, 63 days for the cryptorchid males, and 60 days for the controls. During adulthood the sexual behavior did not

TABLE 1. MEASURES OF SEXUAL BEHAVIOR OF CRYPTORCHID, INTACT, AND CASTRATE MALE GUINEA PIGS EXPRESSED IN AVERAGE PER ANIMAL PER TEST

	Number		Mounts	Intromissions	Ejaculations	Latency to ejaculation in minutes	Scores
	Animals	Tests					
85–120 days-of-age							
Cryptorchid	5	25	3.4	2.4	0.7	4.0	8.6
Intact	5	25	3.2	3.8	0.9	4.3	10.2
Castrate	2	10	1.7	0.0	0.0		2.8
300–335 days-of-age							
Cryptorchid	5	25	6.5	2.6	0.6	4.3	8.7
Intact	5	25	3.5	6.0	0.7	5.9	9.0
730–765 days-of-age							
Cryptorchid	5	25	6.1	2.1	0.5	5.0	8.1
Intact	5	25	6.4	3.2	0.7	6.0	8.8
1095–1130 days-of-age							
Cryptorchid	5	25	6.6	0.2	0.1	7.6	6.0
Intact	5	25	7.5	0.6	0.2	5.9	6.7
Castrate	2	10	1.1	0.0	0.0		4.0

differ significantly (Table 1). The gradual decrease in vigor was no more rapid in one group than in the other and is assumed to have been an effect of age (18).

The appearance of the tissues responsive to the male hormone confirms the conclusion reached from the data on behavior, that the endocrine capacity of the abdominal testis, if reduced at all, is not reduced to a subthreshold level. The epithelium of the epididymides, seminal vesicles, and prostates was similar to that in the controls and very different from that in the castrates (Figs. 6 to 13). Gross measurements of the seminal vesicles and penes were also similar in the two groups, and considerably greater than in the castrates (Table 2). When examined microscopically, the pituitaries from the three groups of males did not appear to be different.

DISCUSSION

Much evidence is found supporting the hypothesis that the endocrine activity of the long-standing abdominal testis is often sufficient for the maintenance of genital tissues in a fully functional state (1, 2, 3, 4, 10).

Additional supporting data have come from this study. Reconciliation of this conclusion with the occurrence of eunuchoidism in many cryptorchid human males is not difficult. The factors responsible for cryptorchidism are multiple (19). Defective testicular anlage (19, 20) and abnormalities in the pituitary-gonadal relationship (21, 22, 23, 24) may be involved. In

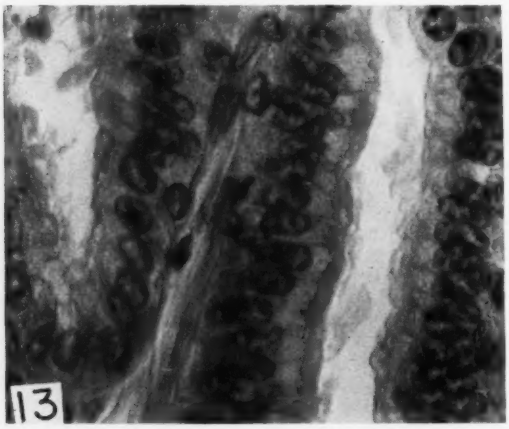

FIG. 13. Prostate epithelium from a cryptorchid guinea pig. ×1000.

such cases interstitial cells might be absent or, if present, non-functional. In other cases the pituitary-gonadal relationship and the gonads are intrinsically normal and the ectopic location of the testes is accounted for by mechanical factors. Cryptorchidism not associated with eunuchoidism (23) probably is of this type and experimentally produced cryptorchidism may be similar. Explanation for the failure of Nelson (17), Moore (10) and ourselves, to obtain similar results cannot be given.

SUMMARY

The effect of cryptorchidism from the time of birth until 3 years of age on the development and maintenance of male sexual behavior, and on the capacity of the testes to secrete hormone in amounts sufficient to maintain the genital tissues has been investigated.

Sexual behavior during the prepuberal period and adulthood was not different in cryptorchid and intact males. The appearance of the epididy-

TABLE 2. AVERAGE WEIGHT AND LENGTH OF SEMINAL VESICLES AND PENES FROM CRYPTORCHID, INTACT, AND CASTRATE GUINEA PIGS

	Cryptorchid	Intact	Castrate
Seminal vesicles (weight)	4.5 gm.	4.7 gm.	0.3 gm.
Seminal vesicles (length)	12.3 cm.	11.8 cm.	5.7 cm.
Penis (weight)	1.5 gm.	1.5 gm.	0.6 gm.
Penis (length)	5.2 cm.	5.5 cm.	4.0 cm.

mis, seminal vesicles, and prostate was similar and very different from that in castrates. It is concluded, therefore, that if the endocrine activity of the testis in the guinea pig was reduced after 3 years of cryptorchidism, the reduction was not sufficient to interfere with the maintenance of the secondary sex characters.

The basis for conflicting clinical observations and experimental results is discussed.

REFERENCES

1. WEBSTER, B.: *J.A.M.A.* **104**: 2157. 1935.
2. REA, C. E.: *Arch. Surgery* **38**: 1054. 1939.
3. BISHOP, P. M. F.: *Guys Hospital Report* **94**: 12. 1945.
4. GROSS, R. E. AND T. C. JEWETT: *J.A.M.A.* **160**: 634. 1956.
5. ENGBERG, H.: *Proc. Roy. Soc. Med.* **42**: 652. 1949.
6. HANES, F. M. AND C. W. HOOKER: *Proc. Soc. Exp. Biol. Med.* **35**: 549. 1937.
7. KIMELDORF, D. J.: *Endocrinology* **43**: 83. 1948.
8. NELSON, W. O.: *Cold Spring Harbor Symposia on Quant. Biol.* **5**: 123. 1937.
9. MOORE, C. R. AND T. F. GALLAGHER: *Am. J. Anat.* **45**: 39. 1930.
10. MOORE, C. R.: *Yale J. Biol. Med.* **17**: 203. 1944.
11. WEBSTER, R. C. AND W. C. YOUNG: *Fertility and Sterility* **2**: 175. 1951.
12. YOUNG, W. C. AND J. A. GRUNT: *J. Comp. & Physiol. Psychol.* **44**: 492. 1951.
13. VALENSTEIN, E. S., W. RISS AND W. C. YOUNG: *J. Comp. Physiol. Psychol.* **47**: 162. 1954.
14. MOSSMAN, H. W., R. A. HOFFMAN AND C. M. KIRKPATRICK: *Am. J. Anat.* **97**: 257. 1955.
15. MOORE, C. R.: *Am. J. Anat.* **34**: 269. 1924.
16. COOPER, R. A.: *J. Anat.* **64**: 5. 1929.
17. NELSON, W. O.: *Recent Progress in Hormone Research* **6**: 29. 1951.
18. GRUNT, J. A. AND W. C. YOUNG: *Endocrinology* **51**: 237. 1952.
19. NELSON, W. O.: *J. Urol.* **69**: 325. 1953.
20. PASCHKIS, K. E., A. E. RAKOFF AND A. CANTAROW: *Clinical Endocrinology*. Paul B. Hoeber Inc., New York. 1954.
21. HESS, J. H. AND R. H. KUNSTADTER: *J. Pediat.* **11**: 324. 1937.
22. MOLITCH, R.: *J. Nerv. & Ment. Dis.* **85**: 51. 1937.
23. CARMICHAEL, H. T. AND A. T. KENYON: *Arch. Neurol. and Psychol.* **40**: 717. 1937.
24. RUBENSTEIN, H. S.: *J. Clin. Endo.* **2**: 519. 1942.

RELATION BETWEEN BIOLOGICAL ACTIVITY OF HYDROCORTISONE ANALOGUES AND THEIR RATES OF INACTIVATION BY RAT LIVER ENZYME SYSTEMS

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RECENT studies show that desaturation of the 1, 2 positions of hydrocortisone and introduction of other substituents (2α-methyl- and 9α-fluoro-groups) lead to increased biological activity (1, 2, 3, 4, 5, 6). On the basis of plasma steroid levels, it has been suggested that the prolonged biological half-life of steroid analogues is due to decrease in rate of metabolism by tissues (5, 7, 8, 9).

Studies were initiated to determine whether elevated plasma levels and increased glycogen deposition activities of hydrocortisone derivatives are related to rate of inactivation in liver. In summary of much work in this area, it may be stated that C-20 and Δ^4-3 ketone reduction of hydrocortisone, as well as ketone reduction of a number of other steroids, occurs in a combined microsome-supernatant fraction of rat liver (10, 11, 12, 13). Hydrocortisone is reduced by rat liver enzyme systems to the corresponding dihydro and tetrahydro derivatives (10). It is also reduced at the C-20 ketone position to both the C-20 alcohol with intact A-ring or further reduced to the corresponding pentol (12, 14). Reduced derivatives of hydrocortisone are subsequently conjugated with glucuronic acid in the liver (15) and removed from the circulation by excretion through the kidney (16). Such reductive steps in the metabolism of hydrocortisone may be referred to as "inactivation" insofar as the dihydro and tetrahydro derivatives and C-20 reduced products (Reichstein's E and the pentol) are inactive in the rat liver glycogen deposition assay. Furthermore, simultaneous administration of these reduced derivatives in combination with hydrocortisone fails to influence the degree of biological response to hydrocortisone in the liver glycogen deposition assay (17).

Presumably, if introduction of substituents prevents reduction of biologically active groups on the steroid nucleus (Δ^4-3 and C-20 ketone groups), such compounds would show increased biological activity merely because of a longer existence in the blood and tissues. It is of considerable importance to be able to determine if steroid analogues exert their increased glycogenic effects by a continuation of an already existing phenomenon,

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or because of a different mechanism brought about by introduction of various substituents on the molecule.

METHODS

The liver glycogen deposition assay has been described previously (18). Multiple dose assay techniques were used to compare glycogenic potency ratios of hydrocortisone and its analogues.

In order to compare rates of liver glycogen deposition as a function of time the 24-hour fasted, adrenalectomized rat was used. Both the analogues and hydrocortisone were administered subcutaneously in 0.2 ml. of carboxymethylcellulose or intravenously in 0.1 ml. of 95% ethanol.

Quantitative assay methods for the evaluation of steroid ketone reduction rates in rat liver fractions have been reported (11, 13, 14). Modifications have been introduced and for purposes of convenience methods will be described in greater detail. Discussion of methods will center around four major points: (1) preparation of the relatively crude microsome-supernatant fractions of rat liver; (2) preparation and composition of the enzyme solution; (3) the enzyme assay; and (4) the extraction and final measurement of steroid substrate disappearance, determined by UV absorption for Δ^4-3 ketone disappearance, and the phenylhydrazine reaction of Porter and Silber (19) for the disappearance of the 17,21-dihydroxyacetone side chain.

1. *Preparation of the enzyme.* Livers were quickly removed from decapitated rats (250–300 gm.) in a +4° C cold room, blotted on filter paper, weighed, and subsequently homogenized in a Potter-Elvehjem type homogenizer with 0.25 *M* sucrose: 0.04 *M* nicotinamide. Whole homogenates of liver were centrifuged at 7,000 g's for 15 minutes in a high speed refrigerated centrifuge. So-called "fluffy layers" and supernatant fractions were removed, recombined, and made to final volumes equal to a 20% w/v mixture of original whole homogenate.

2. *The enzyme solution.* This preparation had the following composition and was stable for long periods when frozen.

Milliliters	*Reagent*
50.0	0.04 *M* $MgCl_2 \cdot 7H_2O$
50.0	0.20 *M* phosphate buffer, pH 7.4
150.0	0.25 *M* sucrose: .04 *M* nicotinamide
40.0	0.03 *M* glucose-6-phosphate
40.0	0.01 *M* TPN
10.0	0.03 *M* trisodium isocitrate

3. *The enzyme assay.* 3.5 ml. of enzyme solution were transferred to tight-fitting, glass-stoppered test tubes (30.0 ml.), placed in an ice bath, and 2.0 ml. of microsome-supernatant fraction transferred to appropriate tubes. All steroid substrates were added to duplicate tubes in equimolar concentrations (0.1 ml. of an 0.01 *M* solution in ethanol). Duplicate sets of tubes with and without added enzyme were incubated at 37.5°C and and at +2–4° C—the latter tubes served as zero controls. Appropriate tissue blanks, without added steroid substrate, were assayed simultaneously.

4. *The extraction and final estimation of steroid disappearance rates.* Following the necessary time intervals, 20 ml. of chloroform or a 50% ethyl acetate:chloroform mixture were added to all tubes, stoppered and agitated in an automatic shaker for 10 minutes. Aqueous layers were removed by suction and discarded. Solvent phases were scrubbed with 0.1N HCl and 0.1N NaOH; aqueous phases removed as before. Five ml. aliquots of solvent phase were transferred in duplicate into appropriate test tubes and dried by filtered air. The above procedures recover 85–104% of all steroids reported below.

Procedures for analysis of disappearance rates of Δ^4-3 ketone and C-20 ketone groups of 17,21-dihydroxy-20-ketosteroids in rat liver fractions have been described. However,

the Allen correction (20) was used in the Porter-Silber method and the equation described by Elliott *et al.* (21) for the ultraviolet analyses. Absorption spectra of individual steroid analogues were determined prior to enzymatic analyses. Ultraviolet absorption spectra varied somewhat between steroid analogues, i.e., 2-methylenehydrocortisone peaked at 260 mμ in the ultraviolet; hydrocortisone at 242 mμ. For this reason, each steroid was analyzed individually using its characteristic absorption factors to quantitate the amount of steroid present in the enzyme mixture.

Quantitative analysis of the rates of disappearance of Δ^1-hydrocortisone analogues was accomplished by means of 3-point analyses applied to their ultraviolet absorption spectra (22).

Results presented in the following sections are divided into three parts: (1) disappearance rates of active glucocorticoids; (2) disappearance rates of inactive glucocorticoids; and (3) studies with 2α-methylcortisone.

RESULTS

1. *Active glucocorticoids.* Figure 1 indicates the rate of Δ^4-3 ketone and C-20 ketone reduction of hydrocortisone as a function of enzyme concentration (incubation time, 2 hours). From these data 400 eq. mg. of rat liver microsome-supernatant fraction were selected for routine enzyme assays.

Figure 2 illustrates the rate of disappearance of the Δ^4-3 ketone and C-20 ketone groups of hydrocortisone as a function of time. Upon analysis of this figure, 15, 45 and 120 minute time intervals were used routinely to follow steroid ketone disappearance rates. It is of interest that the rate of C-20 ketone disappearance is faster than the rate of Δ^4-3 ketone disappear-

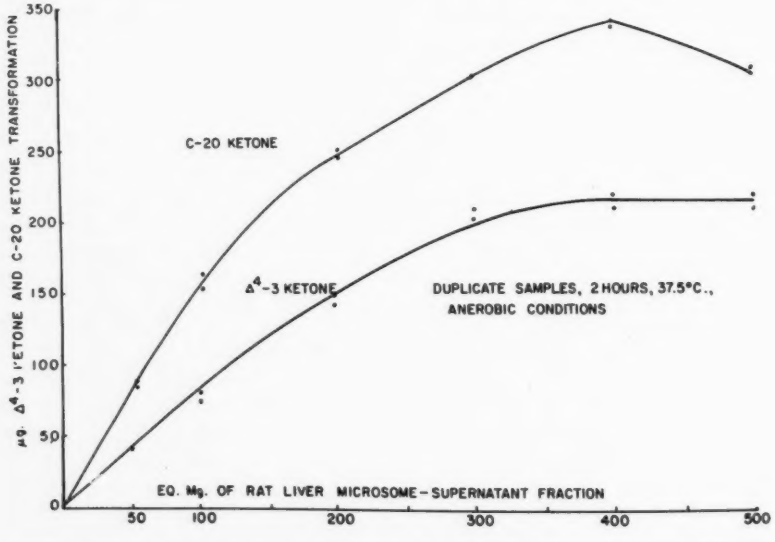


FIG. 1. Rate of disappearance of Δ^4-3 ketone and C-20 ketone groups of hydrocortisone as a function of enzyme concentration.

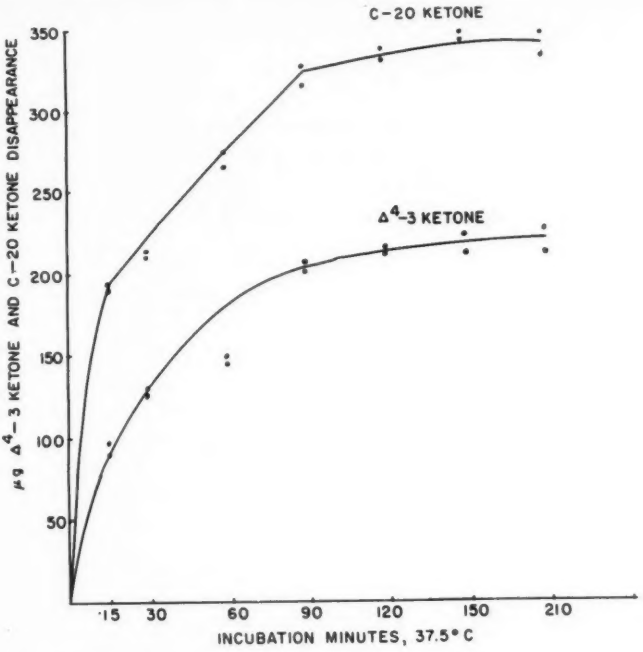
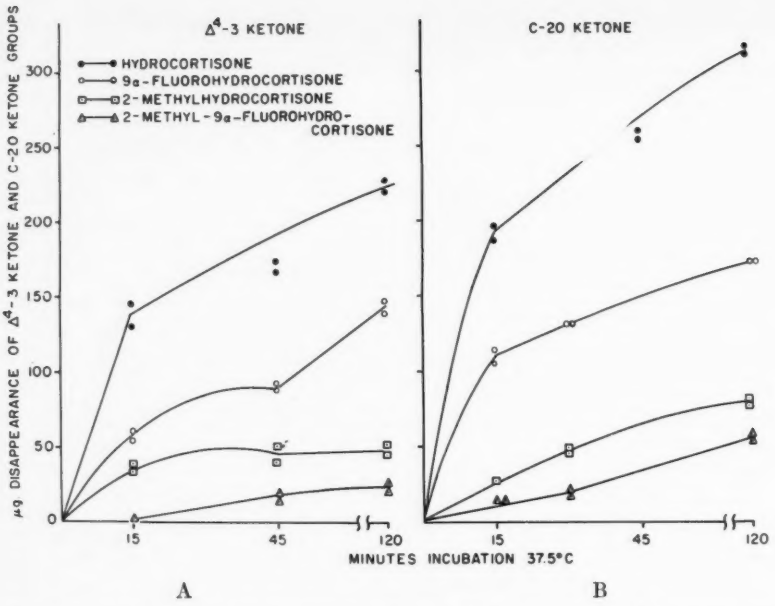


FIG. 2. Rate of Δ^4-3 ketone and C-20 ketone disappearance as a function of time.

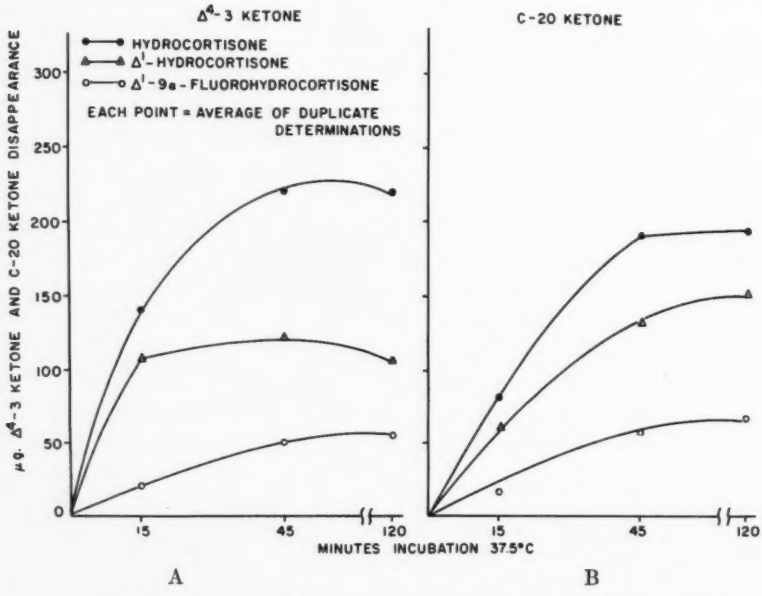
ance. Additional studies have revealed that alterations in co-substrate and co-factor composition are capable of reversing this process, i.e., Δ^4-3 ketone disappearance rates can be made to proceed at faster rates than C-20 ketone disappearance rates. These experimental observations are being investigated more extensively.

In Figures 3A and 3B the disappearance rates of the Δ^4-3 ketone and C-20 ketone positions of hydrocortisone are compared with the rates of disappearance of 9α-fluorohydrocortisone, 2α-methylhydrocortisone and 2α-methyl-9α-fluorohydrocortisone. These compounds were chosen because, in the liver glycogen deposition assay, they were twelve times, six times and thirty-two times as active as hydrocortisone when administered subcutaneously to fasted, adrenalectomized rats (23, 24). Figures 3A and 3B clearly indicate that the rates of disappearance of these steroid analogues are considerably slower than the corresponding rates for hydrocortisone.

In Figures 4A and 4B are illustrated the disappearance rates of hydrocortisone compared to two more of its active analogues—Δ^1-hydrocortisone and Δ^1-9α-fluorohydrocortisone. These two analogues are three times and forty-two times as active as hydrocortisone in causing liver glycogen deposition in fasted, adrenalectomized rats by subcutaneous administration.



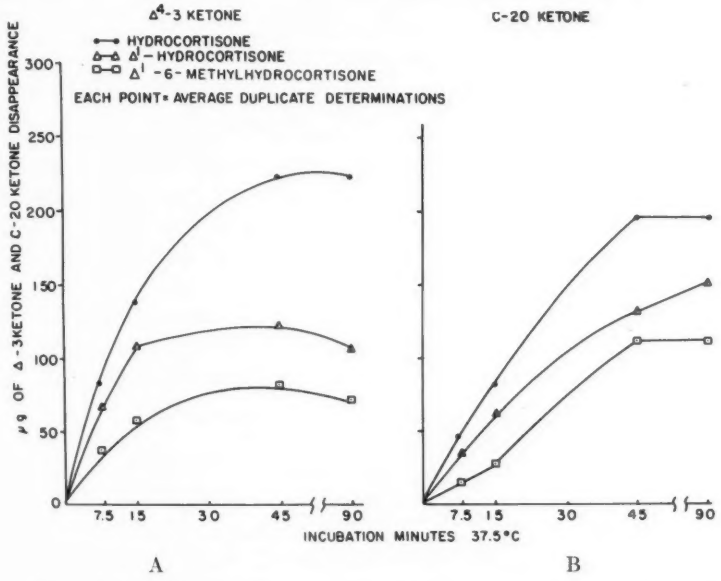
FIGS. 3A and 3B. Rate of disappearance of Δ^4-3 ketone and C-20 ketone groups of hydrocortisone, 2α-methylhydrocortisone, 9α-fluorohydrocortisone, and 2α-methyl-9α-fluorohydrocortisone. (This figure and subsequent figures were drawn before exact position of 2α-methyl and 6α-methyl groups were known.)



FIGS. 4A and 4B. Disappearance rates of the Δ^4-3 and C-20 ketone groups of hydrocortisone, Δ^1-hydrocortisone and Δ^1-9α-fluorohydrocortisone.

Figures 4A and 4B clearly show that the rate of disappearance of the Δ^4-3 ketone and C-20 ketone positions of the analogues are significantly slower than the disappearance rates of these functions on the hydrocortisone molecule.

In Figures 5A and 5B are illustrated the disappearance rates of Δ^4-3 ketone and C-20 ketone positions of Δ^1-6α-methylhydrocortisone, a new derivative of Δ^1-hydrocortisone. This analogue is 8-10 times as active as hydrocortisone in the liver glycogen deposition assay when administered



FIGS. 5A and 5B. Disappearance rates of Δ^4-3 ketone and C-20 ketone groups of hydrocortisone, Δ^1-hydrocortisone and Δ^1-6α-methylhydrocortisone.

subcutaneously (25). The rate of disappearance of both the Δ^4-3 ketone and C-20 ketone positions of this analogue are slower than the corresponding rates for both hydrocortisone and Δ^1-hydrocortisone.

Data presented above suggest that the introduction of substituents on the molecule not only slows down the reduction rate of one end of the steroid molecule, but also interferes with the rate of reduction of substituents on the opposite end of the steroid nucleus. This latter observation agrees with experiments *in vivo* concerning lack of biological activity of tetrahydro derivatives and 20-ketone reduced derivatives (Reichstein's E) of hydrocortisone in the rat liver glycogen deposition assay (17). Similarly, the Δ^4-3 and C-20 ketone reduced derivatives of steroid *analogues* are either inactive or considerably less active than the parent analogues by the same biological methods of analysis (23).

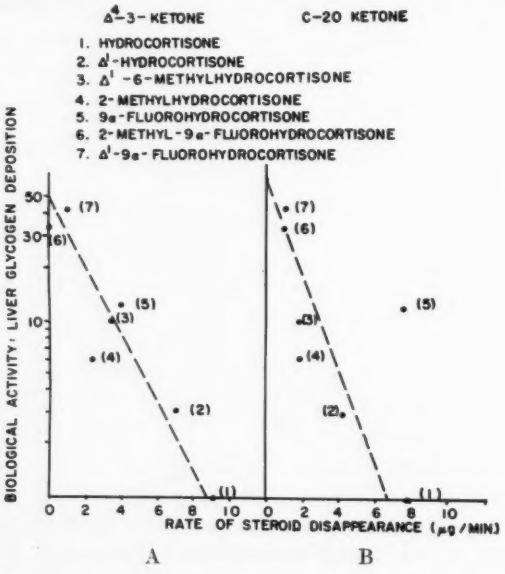
TABLE 1. COMPARISON BETWEEN BIOLOGICAL ACTIVITY OF STEROID ANALOGUES AND DISAPPEARANCE RATES IN RAT LIVER MICROSOME-SUPERNATANT FRACTIONS

Steroid	Potency ratio*	Steroid disappearance† (μg./min.)	
		Δ^4-3 ketone	C-20 ketone
Hydrocortisone	1.0	9.2	7.9
Δ^1-Hydrocortisone	3.0	7.0	4.0
Δ^1-6α-Methylhydrocortisone	10.0	3.5	1.9
2α-Methylhydrocortisone	6.0	2.3	1.7
9α-Fluorohydrocortiosne	12.0	4.0	7.4
2α-Methyl-9α-fluorohydrocortisone	33.0	0	1.0
Δ^1-9α-Fluorohydrocortisone	42.0	1.26	1.0

* Potency ratios are calculated by comparison with hydrocortisone (subcutaneous administration).

† Derived from Figs. 3A, 3B, 4A, 4B, 5A and 5B.

Attempts were made to correlate reduction rates of hydrocortisone and its more potent analogues (Table 1 and Figs. 6A and 6B) with the relative potency ratios of these compounds in the liver glycogen deposition assay. Hydrocortisone was arbitrarily given a potency ratio of 1.0, since it is the parent steroid of all the derivatives studied here. Rates of disappearance (μg./min. during the first 15-minute incubation period, derived from Figs. 3A and 3B, 4A and 4B, and 5A and 5B) are plotted against the log of relative activities of the steroid analogues in the glycogen deposition assay. Disappearance rates of C-20 and Δ^4-3 ketone positions of hydro-



FIGS. 6A and 6B. Correlation between biological activity (liver glycogen deposition) and disappearance rates of steroid analogues in rat liver microsome-supernatant fractions.

cortisone are the averages of Figures 3A, 3B, and 4A and 4B. Although a definite "straight line" relationship does not exist, these two end-points show a remarkable correlation when the multitude of variables contributing to both types of assay are considered.

Previous observations (Figs. 3A and 3B) indicated that the Δ^4-3 ketone and C-20 ketone positions of 2α-methyl-9α-fluorohydrocortisone were reduced at a very slow rate by rat liver enzyme systems. If increased glycogenic activity of this steroid analogue were due to its resistance to enzymat-

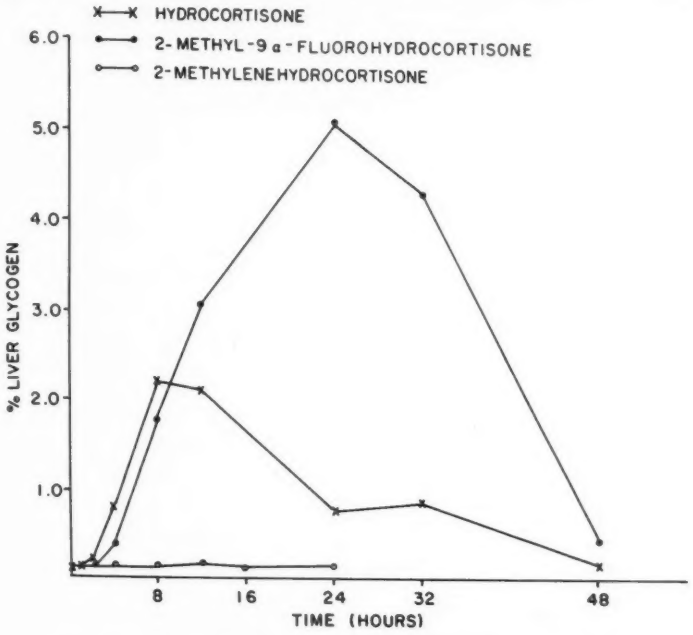


FIG. 7. Liver glycogen deposition following subcutaneous administration of 2α-methyl-9α-fluorohydrocortisone, hydrocortisone and 2-methylenehydrocortisone in the fasted, adrenalectomized rat.

ic attack in the liver, one could reasonably expect that the liver glycogen deposition response will not only be greater, but also persist longer, than the same type of response to equimolar quantities of the parent steroid (hydrocortisone). Figure 7 indicates data obtained when comparisons were made between liver glycogen deposition rates in response to 1.0 mg. of hydrocortisone and 1.0 mg. of 2α-methyl-9α-fluorohydrocortisone by subcutaneous administration. It is readily apparent that liver glycogen deposition is not only greater but persists longer in response to 2α-methyl-9α-fluorohydrocortisone than to hydrocortisone.

Slower rates of absorption from the injection site as well as slower rates of liver metabolism may account for the type of time response shown in

Figure 7. In order to eliminate the question of absorption, experiments were designed to determine if intravenously administered 2α-methyl-9α-fluorohydrocortisone would show a prolongation of the liver glycogen deposition effect. Hydrocortisone (1.0 mg.) and 2α-methyl-9α-fluorohydrocortisone (1.0 mg.) were administered via the tail vein. Livers were removed and analyzed for liver glycogen at various time intervals. It is clearly evident from Figure 8 that equimolar quantities of 2α-methyl-9α-fluorohydrocortisone resulted in a more prolonged, as well as a greatly

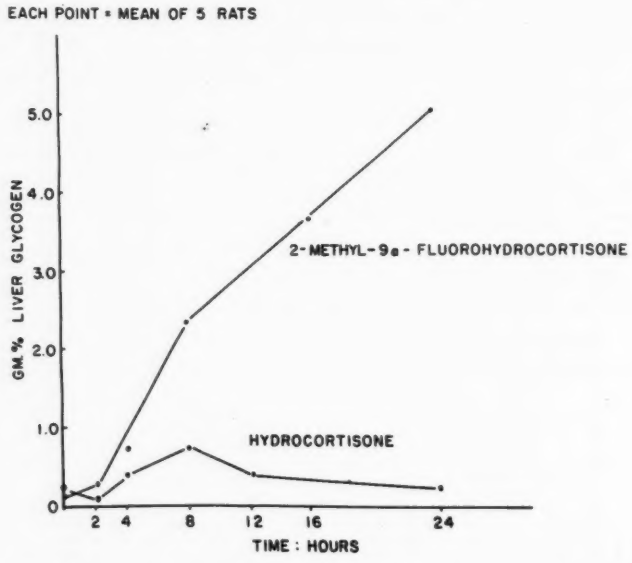


FIG. 8. Liver glycogen deposition response following intravenous administration of hydrocortisone and 2α-methyl-9α-fluorohydrocortisone (1 mg. each).

increased, liver glycogen deposition response. These data suggest that prolongation of liver glycogen deposition seen after intravenous injection is due to a longer existence of the analogue in the blood or tissues of the adrenalectomized rat.

In order to further analyze the situation with regard to liver inactivation, the following experiments were undertaken. One mg. of hydrocortisone and 1.0 mg. of 2α-methyl-9α-fluorohydrocortisone were administered via the portal vein to fasted, adrenalectomized rats. Livers were removed 7 hours after injection and analyzed for liver glycogen. Analysis of the data in Figure 9 indicates that portal-venous administration of 2α-methyl-9α-fluorohydrocortisone caused liver glycogen deposition. Conversely, administration of equal quantities of hydrocortisone via the portal vein did not result in liver glycogen deposition. These data are interpreted to mean

that portal-venous administration of the steroid analogue resulted in less inactivation and more steroid escaped the liver for peripheral tissue utilization.

2. *Steroid analogues inactive as glucocorticoids.* Studies were next undertaken to determine if the lack of glycogenic activity of other steroid analogues could possibly be related to their inactivation rates in the liver. In most instances, the free alcohols of these analogues were employed.

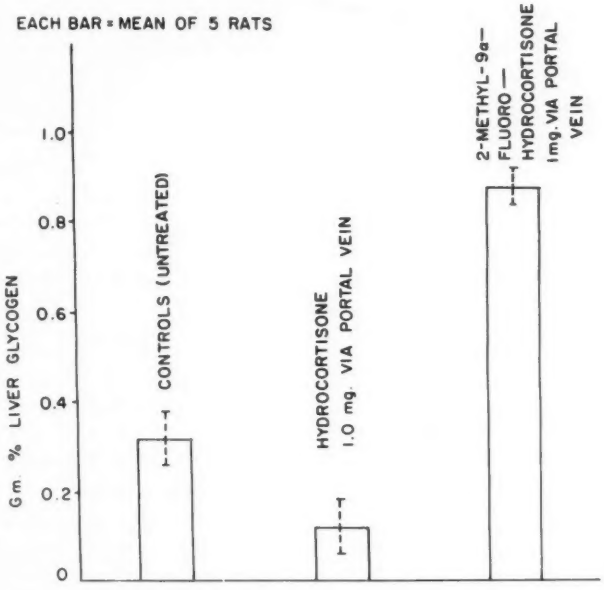
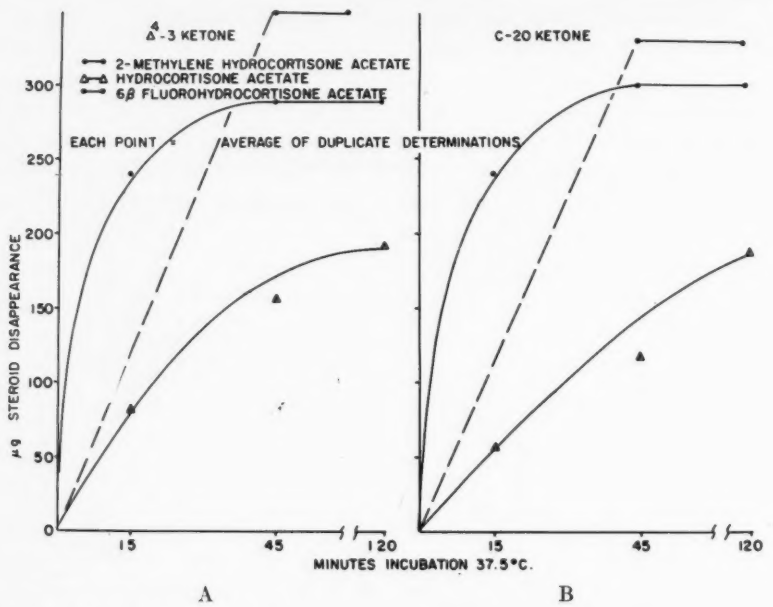


FIG. 9. Liver glycogen deposition response following portal-venous administration of 1.0 mg. hydrocortisone and 2α-methyl-9α-fluorohydrocortisone (ranges are mean variation).

However, when only the acetates were available, disappearance rates were compared with hydrocortisone acetate. Inactive glycogenic steroids which were assayed were: 2-ethylhydrocortisone, 6β-fluorohydrocortisone, 2-methylenehydrocortisone, 19-norhydrocortisone, and 2α-methylcortisone.

In Figures 10A and 10B the reduction rates of 2-methylenehydrocortisone acetate and 6β-fluorohydrocortisone acetate are compared with hydrocortisone acetate. This figure clearly indicates that the two inactive steroid analogues were reduced at faster rates than the parent hormone. It appeared that lack of glycogenic activity of these analogues could be explained, at least in part, on the basis of their rapid inactivation rates in the liver. However, under identical conditions, 2-ethylhydrocortisone and 19-



FIGS. 10A and 10B. Rate of disappearance of Δ^4-3 ketone and C-20 ketone functions of hydrocortisone, 2-methylenehydrocortisone and 6β-fluorohydrocortisone.

norhydrocortisone (inactive as glucocorticoids) were reduced at the Δ^4-3 ketone and C-20 ketone positions at much *slower* rates than the parent hormone (Table 2).

Explanations other than simple liver inactivation rates must be invoked in attempts to explain these latter results. Such attempted explanations would be presumptious on the basis of the limited data. Nevertheless, it is not difficult to imagine that removal of the 19-methyl group and introduction of a 2α-ethyl group on the steroid molecule interferes not only with the biological activity of the steroid molecule at its site of action, but also with the ability of liver enzymes to reduce the Δ^4-3 and C-20 ketone positions. Effects of this type may be of a stereochemical origin and require more extensive investigation.

TABLE 2. RATE OF DISAPPEARANCE OF Δ^4-3 KETONE AND C-20 KETONE FUNCTIONS OF HYDROCORTISONE, 2-ETHYLHYDROCORTISONE, AND 19-NORHYDROCORTISONE

Steroid	Rate of steroid disappearance* μg./min. (First 45-minute incubation period)		
	Δ^4-3 ketone	C-20 ketone	Activity
F Acetate	6.60	6.76	100%
2-Ethyl F Acetate	00	1.84	<10%
19-Nor F Acetate	1.75	3.90	<10%

* Average of duplicate determinations.

Rapid rates of disappearance of Δ^4-3 and C-20 ketone positions of 2-methylene-hydrocortisone led to the following type of analysis: perhaps 2-methylenehydrocortisone did possess glycogenic activity, but its effect was over by the time the liver was ordinarily removed for liver glycogen assay (7 hours). One mg. of 2-methylenehydrocortisone was thereupon injected subcutaneously into adrenalectomized, fasted rats and their livers removed for liver glycogen analysis 1, 2, 4, 8, 16 and 24 hours after injection. Liver glycogen deposition response to this steroid remained negative throughout the experiment (bottom of Fig. 7).

3. *Studies with 2α-methylcortisone.* This steroid analogue is of particular interest because, on the one hand, it is biologically inactive as a "glucocorticoid" and on the other, it differs from the corresponding analogue of hydrocortisone (2α-methyl F) which is six times more active than the parent hormone. Furthermore, the parent hormone of 2α-methylcortisone (cortisone) has about one-half the relative potency ratio in the liver glycogen deposition assay as does hydrocortisone. It was previously observed (Fig. 3A and 3B) that the Δ^4-3 ketone and C-20 ketone reductions of 2α-methyl analogues of hydrocortisone were considerably slower than the corresponding rates for the parent hormone. These data suggested that one possible explanation for the lack of glycogenic activity of 2α-methylcortisone was due either to lack of, or a reduced rate of, conversion to the corresponding 2α-methylhydrocortisone. That is, the introduction of the 2α-methyl group may conceivably interfere with the reduction of the 11-ketone position to the corresponding 11β alcohol. Experiments designed to check this hypothesis are detailed below.

Rat liver microsome-supernatant fractions and co-factors, described in the methods section, were used in the following studies. Cortisone conversion to hydrocortisone was followed by incubating 1.8×10^{-3} *M* cortisone at 37.5°C. for 15, 45 and 90 minutes. Samples were extracted with 50% ethyl acetate: chloroform, and dried under filtered air. Appropriate aliquots were placed on paper strips and chromatographed in the Bush (26) system. Observations under the ultraviolet lamp revealed the presence of a spot which corresponded to the Rf value for hydrocortisone. Small quantities of more polar UV-absorbing compounds were detected but were not analyzed further. Areas on the paper strips corresponding to hydrocortisone were eluted with methanol and analyzed by means of the Porter-Silber method and ultraviolet absorption. The 15, 45 and 90 minute samples contained 20, 25 and 28 μg of hydrocortisone-like material. Amounts of hydrocortisone recovered corresponded to 5.5, 6.9 and 7.8% conversion of cortisone to hydrocortisone during these time intervals. For more positive identification, additional experiments were undertaken using the 45-minute incubation period. Spots corresponding to hydrocortisone were eluted, acetylated with acetic anhydride:pyridine for 16 hours at room temperature, and rechromatographed separately, as well as in combina-

tion with hydrocortisone acetate. Single chromatograms and mixed acetates revealed that the Rf values were identical.

Incubation of 2α-methylcortisone in the same system revealed the presence of a slightly visible area corresponding to 2α-methylhydrocortisone. However, amounts of this material present were too small for accurate quantitation or identification by either the ultraviolet or Porter-Silber methods of analysis. Although numerous explanations may exist, these data suggest that one possible explanation for lack of glycogenic activity of 2α-methylcortisone is its failure to be converted to the corresponding 11β-hydroxylated derivative in the liver.

DISCUSSION

Studies presented above indicate that a fairly close inverse relationship exists between liver glycogen deposition activity of hydrocortisone analogues and their rates of inactivation by rat liver enzyme systems. However, studies with inactive glucocorticoids failed to show the same type of relationship. Possible reasons for these obvious differences cannot be given on the basis of the limited data. Experiments of a more extensive nature are being conducted with other interesting steroid analogues in an attempt to understand these differences.

Whether steroid degradation, as indicated by disappearance rates in isolated systems, bears a causal relationship to steroid action remains unanswered. However, on the basis of the available data, such a possibility appears unlikely, since more active steroid analogues are metabolized at markedly slower rates than hydrocortisone in rat liver enzyme systems. Studies of this type do not entirely preclude the possibility that adrenocortical steroid metabolism in tissues other than liver may bear a direct relationship to steroid action. Such work remains for future investigation.

Although the data which have been presented suggest that a slower rate of metabolism by the liver may be responsible for the increased glycogenic activity of hydrocortisone analogues, they do not explain the well-known observation that some steroid analogues possess properties different from those of the parent hormone. For example, 2α-methyl-9α-fluorohydrocortisone is 32 times as active as the parent hormone in glycogenic activity. However, in direct contrast to the parent hormone, it also causes sodium retention in adrenalectomized, salt-loaded rats. Until direct effects of adrenocortical steroid hormones can be demonstrated on isolated enzyme systems, compatible with their effects in the whole animal, definitive evidence that synthetic steroid derivatives are essentially "more active" or "less readily metabolized" cannot be had.

More experimental facts are needed to ascertain the relative importance of the metabolism of steroid hormones in liver compared to their metabolism in other tissues. If liver metabolism rates partially control the degree of biological response of hydrocortisone analogues, it may be expected that rates of metabolism of naturally occurring glucocorticoids would also

influence their relative activities. Recent data (27) show that corticosterone (a less active glycogenic agent than hydrocortisone) is metabolized in rat liver microsome-supernatant preparations at a significantly faster rate than hydrocortisone. Experiments of this type may help explain, in part, the relative glucocorticoid activities of corticosterone and hydrocortisone.

Studies presented here indicate that the liver plays a significant role in the regulation of both the degree and duration of the biological response to a steroid hormone. Experiments for the future must determine the extent to which various processes, i.e., disease and alterations of the internal and external environments, modify the ability of the liver to affect changes on the steroid molecule. Such experiments should be correlated with changes in biological activities of the various steroids. Final elucidation of these mechanisms should increase our knowledge regarding the manner by which steroid hormones exert their biological effects.

Of particular interest with regard to steroid analogues is the *in vivo* observation that 2α-methylcortisone is inactive in the liver glycogen deposition assay. Studies reported here suggest that one possible explanation for lack of glycogenic activity of this steroid analogue is the relative inability of liver enzyme systems to convert 2α-methylcortisone into the corresponding 11β-hydroxylated derivative which is biologically very active.

Many steroid analogues are currently available. Selections listed here are only a small representative group of the biologically interesting steroid derivatives. More intensive investigations of the above type are being conducted *in vivo* and *in vitro* in attempts to increase our knowledge regarding the potentiation or lack of potentiation brought about by changes introduced on the steroid molecule.

CONCLUSIONS

The relationship between glycogenic activity of hydrocortisone analogues *in vivo* and their rates of inactivation in rat liver enzyme systems *in vitro* has been investigated. Preliminary data show that rates of reduction of Δ^4-3 and C-20 ketone positions of 2α-methylhydrocortisone, 2α-methyl-9α-fluorohydrocortisone, 9α-fluorohydrocortisone, Δ^1-hydrocortisone, Δ^1-6α-methylhydrocortisone, and Δ^1-9α-fluorohydrocortisone are significantly slower than the corresponding rates of reduction of the parent hormone. A fairly close correlation was found to exist between increased biological activity (liver glycogen deposition) and the increased resistance of these analogues to enzymatic attack in the liver.

Preliminary studies with hydrocortisone analogues *inactive* as glucocorticoids (2α-ethyl, 2-methylene, 6β-fluoro, and 19-nor hydrocortisone) revealed that such a simple relationship does not necessarily exist for these derivatives. These apparent differences are discussed.

Data are presented which indicate that one possible explanation for the inactivity of 2α-methylcortisone as a glucocorticoid is the relative inability

of rat liver enzyme systems to convert this compound to the corresponding 11β-hydroxylated derivative.

Although data presented here do not answer all questions pertaining to increased biological activities of steroid analogues, they form the basis for the hypothesis that increased glycogenic activity of steroid analogues may well result from a slower rate of metabolism by the liver.

Acknowledgment

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REFERENCES

1. Thorn, G. W., A. E. Renold, W. I. Morse, A. Goldfien and W. J. Reddy: *Ann. Int. Med.* **43**: 979. 1955.
2. Perlman, P. L. and S. Tolksdorf: *Fed. Proc.* **14**: 377. 1955.
3. Bunim, J. J., M. M. Pechet and A. J. Bollett: *J. Am. Med. Assoc.* **157**: 311. 1955.
4. Spies, T. D., R. E. Stone, G. G. Lopez, C. M. D. Tellechea, R. L. Toca, A. Reboredo and R. M. Svarez: *J. Am. Med. Assoc.* **159**: 645. 1955.
5. Liddle, G. W. and J. E. Richard: *Science* **123**: 324. 1956.
6. Byrnes, W. W., L. E. Barnes, B. J. Bowman, W. E. Dulin, E. H. Morley and R. O. Stafford: *Proc. Soc. Exp. Biol. & Med.* **91**: 67. 1956.
7. Collins, E. J., A. A. Forist and E. B. Nadolski: *Proc. Soc. Exp. Biol & Med.*. **93**: 369. 1956.
8. Ely, R. S., A. K. Done and V. C. Kelley: *Proc. Soc. Exp. Biol. & Med.* **91**: 503. 1956.
9. Silber, R. H. and E. R. Morgan: *Clin. Chem.* **2**: 170. 1956.
10. Tomkins, G. and K. J. Isselbacher: *J. Am. Chem. Soc.* **76**: 3100. 1954.
11. Glenn, E. M. and R. O. Recknagel: *Proc. Soc. Exp. Biol. & Med.* **89**: 153. 1955.
12. Hubener, H. J., P. K. Fukushima and T. F. Gallagher: *J. Biol. Chem.* **220**: 499. 1956.
13. Recknagel, R. O. and E. M. Glenn: *Proc. Soc. Exp. Biol. & Med.* **89**: 156. 1955.
14. Glenn, E. M., P. M. L. Siu and R. O. Recknagel: *Proc. Soc. Exp. Biol. & Med.* **91**: 332. 1956.
15. Isselbacher, K. J. and J. Axelrod: *J. Am. Chem. Soc.* **77**: 1070. 1955.
16. Bongiovanni, A. M., W. R. Eberlein, M. M. Grumbach, J. J. VanWyk and G. Clayton: *Proc. Soc. Exp. Biol. & Med.* **87**: 282. 1954.
17. Glenn, E. M.: Unpublished data.
18. Stafford, R. O., L. E. Barnes, B. J. Bowman and M. M. Meinzinger: *Proc. Soc. Exp. Biol. & Med.* **89**: 371. 1955.
19. Porter, C. C. and R. H. Silber: *J. Biol. Chem.* **185**: 201. 1950.
20. Allen, W. M.: *J. Clin. Endo.* **10**: 71. 1950.
21. Elliott, F. H., W. K. Birmingham, A. V. Schally and E. Schonbaum: *Endocrinology* **55**: 721. 1954.
22. Johnson, J. L. and J. E. Stafford: Unpublished data.
23. Stafford, R. O. and S. C. Lyster: Unpublished data.
24. Lyster, S. C.: Unpublished data.
25. Lyster, S. C., L. E. Barnes, G. H. Lund, M. M. Meinzinger and W. W. Byrnes: *Proc. Soc. Exp. Biol. & Med.* **94**: 159. 1957.
26. Bush, I. E.: *Ciba Foundation Colloquia* 203. 1955.
27. Glenn, E. M. and B. J. Bowman: Unpublished data.

EFFECTS OF HYPOPHYSECTOMY AND HORMONES ON SULFHYDRYL CONCENTRATIONS IN RAT KIDNEY CELLS[1]

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ANY change in water and electrolyte balance produced by a direct effect on renal tubular cells is related to alterations in tubular reabsorptive or secretory mechanisms. It has been demonstrated that mercurial diuresis occurs concomitantly with, and possibly as a result of, a depression in concentration of sulfhydryl groups in cells of terminal portions of the proximal tubules, loops of Henle, and collecting ducts (7, 8). Hormones which influence the state of renal tubular function might do so by affecting the concentration of sulfhydryl groups within renal cells and the studies included in this report were designed to test this hypothesis. A preliminary report has been published (6).

MATERIALS AND METHODS

Hypophysectomized albino, Sprague-Dawley rats (purchased from Hormone Assay Laboratories, Chicago, Ill.) weighing 80–100 gm. were used along with controls of the same age weighing 166–204 gm. The animals were kept in a room maintained at approximately 27° C and were fed a canned meat diet *ad libitum*. Twenty-four days after hypophysectomy the animals were divided into six groups and treated as follows, the daily doses of hormones being expressed per 100 gm. of body weight: group 1, 1 mg. purified growth hormone (GH);[3] group 2, 20 milliunits pitressin; group 3, 5μg of thyroxine; group 4, 0.5 mg. thyrotropin (TSH);[3] group 5, 1 mg. desoxycorticosterone acetate (DCA); group 6 1 cc. isotonic saline. Normal control rats of the same age were also injected with saline. The doses were divided and the rats were weighed and injected intraperitoneally twice each day. Treatment was continued for seven days and on the morning of the eighth day the animals were anesthetized with pentobarbital (30 mg. per kg.). The kidneys were perfused with a 10% trichloracetic acid fixing solution and removed. Slices 2–3 mm. thick were excised from each left kidney from the region midway between the upper and lower poles. The slices were immediately placed in the fixing fluid. The tissue was then dehydrated in ethyl alcohol, cleared in xylol, and embedded in paraffin. Histological sections were prepared and stained for protein-bound sulfhydryl groups using a modification of the method of Barnett and Seligman (1). Right kidneys were also fixed, dehydrated, and cleared in xylol. They were then air-

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[3] Purified growth hormone (Armour lot 369–202B) and thyrotropin (Armour lot R377152) kindly supplied by Dr. I. Bunding of the Armour Laboratories, Chicago, Ill.

dried at room temperature for 1 hour and weighed on a torsion balance. Relative concentration of protein-bound sulfhydryl groups were measured in the cytoplasm of renal cells using a microspectrophotometer. Sections were scanned and cells which had intact membranes and whole nuclei were measured. Ten to twenty different cells of a given type were measured in each section. Details of the entire procedure for preparing tissues, staining for SH groups and measuring their concentration can be found in a previous publication (9).

RESULTS

Growth hormone treatment re-established growth of hypophysectomized rats (Table 1). Untreated hypophysectomized rats and those which received Pitressin, thyroxin, TSH or DCA gained only 5–10 gm. during the 7-day treatment period but animals injected with growth hormone during the same interval gained an average of 33 gm. Control animals of the same

TABLE 1. EFFECTS OF VARIOUS HORMONES ON BODY AND KIDNEY WEIGHTS OF HYPOPHYSECTOMIZED (HPX.) RATS. (SEE TEXT FOR METHOD OF PREPARING TISSUES PRIOR TO WEIGHING)

Group	No. of animals	Average increase in body weight (gm.)*	Average right kidney weight (mg.)*
Controls	8	52 ±2	242 ±9
Hpx.	12	5 ±1	89 ±2
Hpx.+GH	12	33 ±1†	105 ±2†
Hpx.+pitressin	6	6 ±1	85 ±3
Hpx.+thyroxin	6	7 ±1	97 ±2
Hpx.+TSH	6	10 ±1	97 ±5
Hpx.+DCA	6	6 ±2	81 ±3

* Standard errors.
† Differ from untreated hypophysectomized group ($P<.01$).

age as the hypophysectomized groups gained an average of 52 gm. but their initial starting weights were higher. The increase in body weight and the difference in kidney weights between growth hormone treated and untreated hypophysectomized animals were significant ($P<.01$).

"Nontreated hypophysectomized rats." Concentrations of protein-bound sulfhydryl groups in the cytoplasm of renal cells of normal and hypophysectomized rats are given in Table 2. Hypophysectomy resulted in a significant depression in sulfhydryl group levels in cells of the straight terminal portions of proximal tubules and in collecting ducts but no changes were observed in cells of the convoluted parts of the proximal and distal tubules or loops of Henle. The deficiencies in affected cells represented approximately a 30–40 per cent reduction from control values (Table 2).

"Growth hormone-treated group." Administration of this hormone over a period of 7 days resulted in partial restoration of sulfhydryl levels in cells of the straight terminal portions of proximal tubules but a similar result was not observed in collecting duct cells (Table 2). The values in collecting

ducts were equivalent to those obtained in the untreated hypophysectomized group.

"*Pitressin-treated group.*" Pitressin administration to hypophysectomized rats resulted in a significant reduction in sulfhydryl groups, in cells of the convoluted portions of the proximal and distal convoluted tubules (Table 2), but no significant alterations occurred in the cells of terminal portions of proximal tubules or medullary collecting ducts.

"*Thyroxin and TSH-treated group.*" Table 2 gives data on the effects on thyroxin and TSH. A slight but not significant depression in sulfhydryl group concentrations occurred in convoluted portions of proximal tubules when these hormones were administered to hypophysectomized rats. In

TABLE 2. EFFECT OF HYPOPHYSECTOMY AND SUBSTITUTION THERAPY ON PROTEIN-BOUND SULFHYDRYL GROUP CONCENTRATION IN THE CYTOPLASM OF RAT KIDNEY CELLS

Treatment	Extinction values*					
	Proximal tubules		Loops of Henle		Distal convoluted tubules	Medullary collecting ducts
	Convoluted portions	Straight portions	Desc. limbs	Asc. limbs		
Controls	.552 ± .010	.407 ± .001	.343	.383	.547 ± .016	.264 ± .003
Hpx.	.534 ± .020	.268 ± .007	.332	.357	.550 ± .005	.176 ± .013
Hpx. + GH	.579 ± .031	.363 ± .005	.334	.358	.543 ± .006	.168 ± .012
Hpx. + pitressin	.359 ± .019	.259 ± .012	—	—	.371 ± .020	.128 ± .018
Hpx. + thyroxin	.502 ± .020	.328 ± .013	—	—	.554 ± .007	.146 ± .025
Hpx. + TSH	.470 ± .011	.392 ± .013	—	—	.540 ± .001	.143 ± .021
Hpx. + DCA	.503 ± .011	.476 ± .008	—	—	.531 ± .015	.142 ± .021

* Means ± standard errors.

addition the low levels characteristically seen in cells of the straight terminal portions of proximal tubules of hypophysectomized rats were raised towards normal levels by thyroxin and especially by TSH (Table 2). In contrast no effects of these hormones were noted in cells of distal convoluted tubules or collecting ducts.

"*DCA-treated group.*" DCA produced a large increase in sulfhydryl group concentrations in cells of the terminal portions of proximal tubules of hypophysectomized animals. The values shown are actually greater than control values and differ significantly from the normal controls ($P < .01$). The effects of DCA in other types of renal cells were not significant (Table 2).

Since cells of the thin and thick portions of the loops of Henle are difficult to analyze for SH groups particularly in the smaller kidneys of hypophysectomized animals, a sufficiently large sample of these cells was studied only in control, hypophysectomized, and hypophysectomized animals treated with growth hormone. The results listed in Table 2 do not reveal any differences between the three groups. A limited number of measurements in the other groups studied indicated that SH concentrations in cells

of this type were relatively stable but the data are not presented since in most instances the samples included only a few animals.

DISCUSSION

The experiments reported on deal with direct effects of hormones on tubular cells, and additionally, focus attention on a specific chemical entity, the sulfhydryl group, which is known to be a constituent of many cellular enzymes (2, 14), necessary for growth and cellular division (5, 12), and may be necessary for hormonal synthesis and activity (2). Our results show that hypophysectomy and hormonal substitution therapy produced changes in protein-bound sulfhydryl groups only in certain types of renal cells. This suggests that various hormones have specific target cells in the kidney and that these specific biochemical changes are probably also related to the functional changes produced by these hormones.

Reductions in clearance and tubular maxima for *p*-aminohippurate, diodrast, and glucose, as well as urea, inulin and creatinine clearances in hypophysectomized animals have been reported by many workers (4, 10, 13, 15, 16). It has been shown (3, 11, 17, 18) that thyroid or growth hormone (essentially free of TSH contamination) can partially restore these functions. The proximal tubule is most likely involved in tubular transport of PAH and diodrast. Growth hormone, thyroxin, and TSH partially re-established transport capacities for these substances and at the same time partially restored sulfhydryl group concentrations in the proximal tubule. This suggests that sulfhydryl groups may be involved in active renal tubular mechanisms.

The effects of Pitressin on sulfhydryl groups of the proximal convoluted and distal convoluted tubules were clear cut. However, the functional significance of these changes defy analysis since hypophysectomy produces a multiplicity of hormonal defects which may be closely interrelated. The Pitressin used probably contained some oxytocic principle and the present findings do not allow separation of effects of either of these hormones.

DCA effects on protein-bound SH of hypophysectomized rats provides further evidence that in some way this radical is concerned with the renal transport of electrolytes.

The findings reported here suggest that the quantitative histochemical approach may be helpful in elucidating some of the biochemical effects of various hormones in specific cells. Much work in this direction is still to be done and a similar approach may be useful in the study of the effects of the adrenals, thyroid and other endocrine organs. Advances in this field are dependent on the development of quantitative histochemical methods for studying a number of cellular constituents and especially enzyme actions.

SUMMARY

Measurements of protein-bound sulfhydryl groups in the cytoplasm of kidney cells of normal, hypophysectomized and hypophysectomized rats treated with different hormones were made using histochemical techniques. Sulfhydryl group concentrations in most renal cell types were found to be normal in hypophysectomized animals but were depressed in cells of the terminal portions of proximal tubules and collecting ducts. Purified growth hormone, thyroxin, and TSH raised the values in cells of the straight terminal proximal portions of tubules of hypophysectomized rats. DCA increased sulfhydryl group concentration well above normal levels. Concentrations in other types of tubular cells were not affected by these hormones. Pitressin decreased sulfhydryl group concentrations in cells of the convoluted portions of proximal and distal convoluted tubules. None of the hormones used could re-establish normal levels of sulfhydryl in collecting duct cells of hypophysectomized rats. It is suggested that the observed changes in concentration of SH groups are related to depression of tubular transport mechanisms which may require protein-bound sulfhydryl groups in order to maintain activity.

REFERENCES

1. Barnett, R. J. and A. M. Seligman: *Science* **116**: 323. 1952.
2. Barron, E. S. G.: *Recent Adv. Enzymol.* **11**: 201. 1951.
3. deBodo, R. C., I. L. Schwartz, J. Greenberg, M. Kurtz, D. P. Earle, Jr. and S. J. Farber: *Proc. Soc. Exper. Biol. Med.* **76**: 612. 1951.
4. Boss, W. R., C. M. Osborn and A. A. Renzi: *Endocrinology* **51**: 66. 1952.
5. Brachet, J.: *Chemical Embryology*, Interscience Publishers, New York, 1950.
6. Cafruny, E. J., E. Carhart, H. S. Di Stefano and A. Farah: *Fed. Proc.* **14**: 323. 1955.
7. Cafruny, E. J. and A. Farah: *J. Pharmacol. & Exper. Therap.* **117**: 101. 1956.
8. Cafruny, E. J., A. Farah and H. S. Di Stefano: *J. Pharmacol. & Exper. Therap.* **115**: 390. 1955.
9. Cafruny, E. J., H. S. Di Stefano and A. Farah: *J. Histochem. & Cytochem.* **3**: 354. 1955.
10. Earle, D. P. Jr., R. C. deBodo, I. L. Schwartz, S. Farber, J. M. Kurtz and J. Greenberg: *Proc. Soc. Exp. Biol. Med.* **76**: 608. 1951.
11. Farah, A., F. Koda and M. Frazer: *Endocrinology* **58**: 399. 1956.
12. Hammett, F. S. and L. Walp: *Growth* **3**: 427. 1939.
13. Luft, R. and B. Sjorgen: *Acta Endocrinologica* **4**: 351. 1950.
14. Olcott, H. S. and H. Fraenkel-Conrat: *Chem. Rev.* **41**: 151. 1947.
15. White, H. L. and P. Heinbecker: *Am. J. Physiol.* **130**: 464. 1940.
16. White, H. L. and P. Heinbecker and D. Rolf: *Am. J. Physiol.* **149**: 404. 1947.
17. White, H. L., P. Heinbecker and D. Rolf: *Am. J. Physiol.* **157**: 47. 1949.
18. White, H. L., P. Heinbecker and D. Rolf: *Am. J. Physiol.* **165**: 442. 1951.

COMPARISON OF THE HYPOGLYCEMIC ACTION OF TOLBUTAMIDE IN THE FOWL AND OTHER SPECIES[1,2]

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THE immediate hypoglycemic response to the aryl sulfonylureas, 1-butyl-3-p-aminobenzene-sulfonylurea (BZ-55, Carbutamide), 1-butyl-3-p-tolylsulfonylurea (U-2043, Tolbutamide, Orinase) and related compounds (e.g., p-amino-sulfonamide-isopropylthiodiazole) is dependent upon the presence of beta cells in the islets of Langerhans of the pancreas of man (1, 2, 3, 4), dog (5, 6, 7), rabbit (8, 9), rat (10, 11) and toad (7). Accordingly, it is generally agreed that irrespective of the precise manner whereby the sulfonylureas exert their hypoglycemic action, the secretion of insulin by the pancreas is involved.

The aforementioned species develop hyperglycemia and other manifestations of diabetes mellitus after ablation or destruction of the islet tissue. Since pancreatectomy does not produce similar changes in the duck or chicken (12, 13), it became pertinent to investigate the effect of the sulfonylureas on the blood sugar of normal and depancreatized fowl.

METHODS

Chickens and Pekin ducks of from 1.5 to 3.0 kg. in weight were used in this study. After an overnight fast, the fowl were given from 50 to 200 mg. tolbutamide[3] per kilogram body weight by stomach tube or by intravenous injection. Venous blood samples were taken at 1 hour, ½ hour and immediately before, and at hourly intervals for from four to six hours after the administration of the sulfonylurea. Similar studies were performed with healthy men, dogs, rabbits and rats. Likewise, alloxanized chickens, alloxanized dogs, alloxanized rabbits, depancreatized ducks and acutely enterectomized ducks were studied. Chickens and rabbits were given an intravenous injection of 200 mg. alloxan monohydrate per kilogram, rats were given a subcutaneous injection of 180 mg./kg., and dogs were given an intravenous injection of 65 mg./kg body weight.

The glucose concentration of the venous samples was determined by Nelson's procedure (14). The mean of the three samples taken before the administration of the tolbutamide was expressed as the initial concentration.

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RESULTS AND DISCUSSION

In accord with previous observations, the fasting blood sugar concentration of the healthy chicken and duck is greater than that of man, dog, rabbit and rat. The effect of various dosages of tolbutamide on the blood sugar of normal chickens and ducks is summarized in Table 1. It is apparent that the hypoglycemic response of the normal fowl to the oral and intravenous administration of the aryl sulfonylurea is essentially the same as that of normal man, dog, rabbit and rat. The only apparent difference between the response of the fowl and that of other species is that the former show a somewhat greater immediate decrease and a more rapid restitution to the initial blood sugar concentration after the intravenous injection of 50 mg. tolbutamide per kilogram of body weight.

Whereas the administration of alloxan results in the development of hyperglycemia in rats, rabbits and dogs, no apparent effect is produced in the chicken. Thus, the blood sugar concentration of a group of five chickens was 184 ± 3.8 mg. % before and 188 ± 5.4 mg. % on the 4th day after the administration of alloxan. These observations are in accord with similar findings in the duck (15).

The administration of tolbutamide to alloxanized chickens results in the same hypoglycemic response as that of the normal chicken while similarly treated alloxanized dogs, rabbits and rats show no significant change in the blood sugar concentration (Table 2).

Like alloxanization, pancreatectomy does not produce an appreciable change in the blood sugar concentration of the fowl (12, 13). Thus the blood sugar of a group of 8 ducks was 172.5 ± 7.7 mg. % before pancreatectomy and 160.8 ± 5.5 mg. % in 16 days after pancreatectomy. The intravenous administration of 50 mg. tolbutamide per kilogram body weight to ducks on the 16th day after pancreatectomy results in essentially the same hypoglycemic response as that observed in normal ducks (Table 2, Fig. 1). This response is in contrast to that observed in depancreatized man, dog, rabbit and toad (1 to 11).

In order to obviate the possibility that the response of the depancreatized duck is due to the presence of accessory pancreatic tissue, a group of six ducks was studied immediately after the removal of the entire gastrointestinal tract. Such enterectomized ducks exhibit the same acute hypoglycemic response to the intravenous injection of 50 mg. tolbutamide per kilogram as the normal fowl (Table 2, Fig. 2). The restitution of the blood sugar concentration, however, is markedly delayed in the enterectomized ducks. That this prolongation of hypoglycemia is not due to hepatic damage is indicated by the fact that the blood sugar concentration is back to the pre-injection level in from 8 to 12 hours. Further, the administration of 25 instead of 50 mg. tolbutamide per kilogram body weight results in a more rapid restitution of the blood sugar concentration of the enterectomized duck (Table 2, Fig. 2).

TABLE 1. EFFECT OF TOLBUTAMIDE ON THE BLOOD SUGAR CONCENTRATION OF VARIOUS SPECIES

Species	No.	Dosage, mg./kg.	Route	Blood sugar – mean mg. per cent ±S.E.					
				0	30	60	120	180	240
Man	50	50	mouth	80.5± 1.5	46.8±3.0	45.6±1.3	53.9± 1.5	56.9± 1.6	57.4± 1.5
	15	100	mouth	80.9± 2.4	46.0±3.9	44.7±3.0	55.6± 2.7	50.5± 3.0	51.3± 2.3
Dog	7	50	vein	74.2± 1.9	45.8±1.7	43.1±3.0	50.3± 4.8	52.6± 2.7	51.0± 2.7
	5	100	mouth	72.4± 3.3	42.4±2.0	36.4±3.9	35.6± 5.2	42.0± 5.4	37.5± 5.1
Rabbit	6	50	vein	76.5± 1.6	59.2±4.3	47.4±3.5	46.8± 3.5	48.0± 3.1	51.8± 4.0
Rat	10	50	mouth	83.3± 2.7	—	50.0±2.6	52.4± 2.4	57.9± 2.8	72.7± 3.7
	15	100	mouth	83.0± 3.0	—	49.5±2.6	37.0± 1.7	54.7± 3.3	59.6± 5.7
	10	200	mouth	77.5± 2.3	—	55.2±2.8	41.0± 1.9	40.5± 2.7	42.7± 3.5
Chicken	6	50	vein	169.7±11.5	92.7±9.8	74.7±8.0	58.8± 4.1	89.0± 9.8	115.2± 8.0
	14	200	mouth	169.5± 3.5	101.1±6.2	50.6±3.8	40.3± 2.3	46.7± 2.6	62.9± 4.9
Duck	14	50	vein	151.3± 6.5	66.9± 8.8	49.2 6.6	75.0±10.5	112.9±14.1	143.2± 14.0

TABLE 2. EFFECT OF TOLBUTAMIDE ON THE BLOOD SUGAR CONCENTRATION OF ALLOXANIZED OR DEPANCREATIZED FOWL AND OTHER SPECIES

Animal	No.	Dosage, mg./kg.	Route	Blood sugar – mean mg. per cent ±S.E.					
				0 min.	30 min.	60 min.	120 min.	180 min.	240 min.
Alloxanized dog	5	50	Vein	248.2±23.7	252.6±21.4	264.0±20.2	261.4±19.7	266.6±20.4	268.0±31.6
Alloxanized rabbit	9	50	Vein	329.0±94.1	339.3±38.4	355.6±32.5	345.4±37.4	335.1±36.6	340.3± 6.1
Alloxanized rat	8	100	Mouth	352.1±17.5	—	343.1±17.5	334.6±20.5	335.5±19.3	336.5±20.5
Alloxanized chicken	5	200	Mouth	188.0± 5.4	107.4± 9.0	58.4±11.0	44.8± 8.3	48.6± 4.9	109.0± 5.2
Depancreatized duck	8	50	Vein	160.8± 5.5	84.1± 9.8	65.6± 7.9	109.5±10.7	136.2±14.3	143.6±18.6
Enterectomized duck	3	25	Vein	176.2±29.5	143.0±25.4	106.7±33.4	108.7±52.8	139.0±43.7	170.3±44.7
Enterectomized duck	6	50	Vein	154.5±13.7	102.0±13.7	69.2±13.6	59.5±17.4	75.2±28.0	88.8±36.6

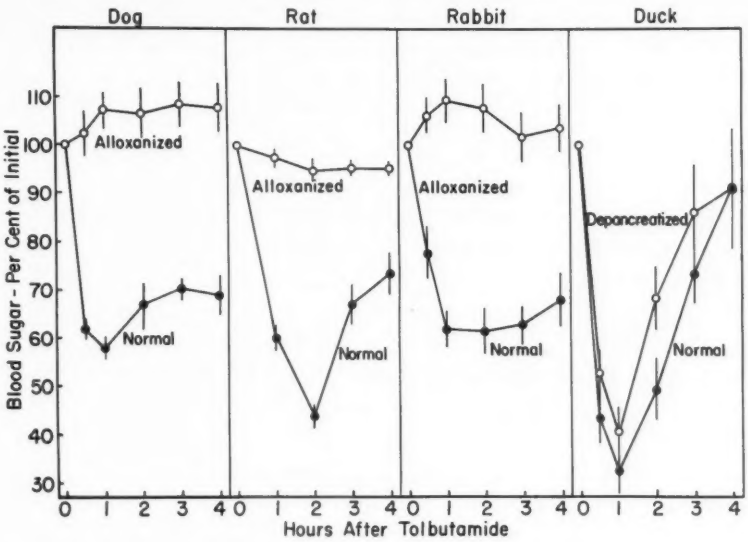

FIG. 1. Effect of tolbutamide on the blood sugar concentration of normal and alloxanized dogs, rabbits and rats and of normal and depancreatized ducks. The blood sugar concentration at each interval is expressed as the mean ±SE of the percentages of the initial concentrations.

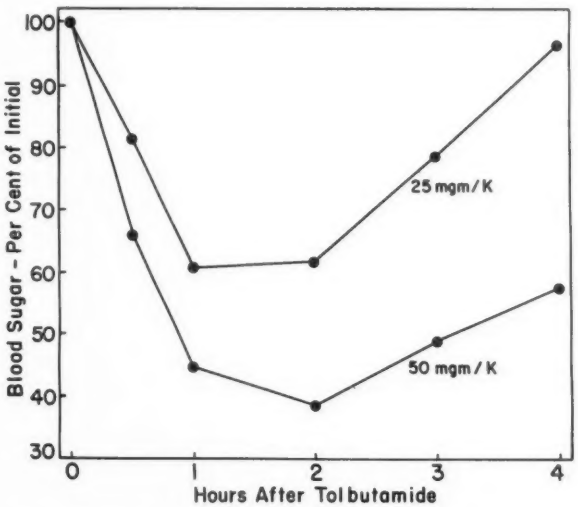

FIG. 2. Effect of tolbutamide on the blood sugar concentration of completely enterectomized ducks. The blood sugar concentration at each interval is expressed as the mean of the percentages of the initial concentration.

The possibility that the liver may serve as a source of insulin in the duck was excluded by studies on the hypoglycemic action of liver extracts prepared according to the technique of Scott and Fisher (16) for the extraction of insulin from the pancreas. Such hepatic extracts exerted no hypoglycemic action.

SUMMARY AND CONCLUSIONS

The administration of tolbutamide by mouth or by vein results in essentially the same hypoglycemic response in man, dog, rabbit, rat, chicken and duck. Whereas alloxanized dogs, rabbits and rats show no hypoglycemic response to tolbutamide, alloxanized chickens, depancreatized ducks and completely enterectomized ducks develop a brisk hypoglycemic response. Accordingly, the hypoglycemic response of the fowl to tolbutamide is independent of the presence of the pancreas and/or the discharge of insulin into the circulation.

REFERENCES

1. Miller, M. and J. W. Craig: *Metabolism* **5**: 162. 1956.
2. Purnell, R., Y. Arai, E. Pratt, C. Hlad, Jr. and H. Elrick: *Metabolism* **5**: 778. 1956.
3. Goetz, F. C., A. S. Gilbersten and V. Josephson: *Metabolism* **5**: 788. 1956.
4. Moorhouse, J. A. and R. M. Kark: *Metabolism* **5**: 847. 1956.
5. Loubatieres, A.: *Compt. rend. Soc. Biol.* **138**: 766. 1944.
6. Fritz, I. B., J. V. Morton, M. Weinstein and R. Levine: *Metabolism* **5**: 744. 1956.
7. Houssay, B. A. and J. C. Penhos: *Metabolism* **5**: 727. 1956.
8. Chen, K. K., R. C. Anderson and N. Maze: *Proc. Soc. Exp. Biol. and Med.* **63**: 483. 1946.
9. Wick, A. N., B. Britton and R. Grabowski: *Metabolism* **5**: 739. 1956.
10. Mirsky, I. A., G. Perisutti and R. Jinks: *Proc. Soc. Exp. Biol. and Med.* **91**: 475. 1956.
11. Miller, W. L., J. J. Krake and M. J. Vander Brook: In press.
12. Minkowski, O.: *Arch. f Exper. Path. u. Pharmakol.* **31**: 85. 1892.
13. Mirsky, I. A., N. Nelson, I. Grayman and M. Korenberg: *Amer. J. Physiol.* **135**: 223. 1941.
14. Nelson, N.: *J. Biol. Chem.* **153**: 375. 1944.
15. Mirsky, I. A.: *Proc. Soc. Exp. Biol. and Med.* **59**: 35. 1945.
16. Scott, D. A. and A. M. Fisher: *J. Clin. Invest.* **17**: 725. 1938.

THE EFFECTS OF THYROXINE AND CERTAIN METAL IONS UPON OXIDATION AND PHOSPHORYLATION *IN VITRO*[1]

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THE exact mechanism of action of the thyroid hormone on cellular metabolism has not been established. The addition of thyroxine or triiodothyronine to media containing portions of *intact* tissues does not accelerate the oxygen uptake of those tissues (1, 2). Under certain experimental conditions *in vitro*, however, thyroxine does influence respiration of cellular material and uncouples oxidative phosphorylation (3–8). It has been demonstrated readily that thyroxine and several analogues of thyroxine augment the oxygen utilization of a rat heart homogenate system (2, 9, 10, 11). The present report describes the actions on respiration and phosphorylation in this system of thyroxine, of magnesium and other cations and of combinations of thyroxine and cations.

MATERIAL AND METHODS

The homogenate was prepared by grinding approximately 620 mg. heart muscle obtained from a normal Sprague-Dawley rat in 9.0 ml. ice cold 0.1 *M* Na_2HPO_4 at pH 7.80 in a glass homogenizer; the material was then centrifuged at 600 *g* for five minutes. The supernatant fluid was employed for experimental purposes; it contained approximately 0.67 mg. total nitrogen per ml. The reaction mixtures in the main compartments of the Warburg flasks contained 0.4 ml. homogenate with 40 μM disodium phosphate, 55 μM Tris buffer at pH 7.80, 0.001 N NaOH with or without 16.5 μM *l*-thyroxine,[3] 3.2 μM sodium salt of adenosinetriphosphate (ATP, Pabst), 0.15 μM cytochrome c (Sigma), and a solution of magnesium chloride, calcium chloride, manganese dichloride, cobaltous chloride, beryllium carbonate, strontium chloride or zinc sulfate. In one side arm were 60 μM sodium succinate at pH 7.80 and 10 mg. hexokinase (Pabst) with 150 μM glucose, in the second side arm 0.2 ml. 20% trichloracetic acid, and in the center well 0.2 ml. 20% KOH with pieces of filter paper. The total volume in each flask was 3.2 ml. The pH of the whole mixture without the trichloracetic acid did not change significantly between the beginning and end of the experiment. Respiration was measured by the conventional Warburg techniques at 37° with air as the gas phase. The flasks were first equilibrated for 10 minutes, after which initial readings were recorded, and the succinate and hexo-

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[3] The sodium *l*-thyroxine was generously supplied through the courtesy of Dr. A. E. Heming of Smith, Kline and French Laboratories.

kinase were tipped into the main chambers. Zero time flasks were removed and the trichloracetic acid was tipped into these. Readings of oxygen utilization in the remaining flasks were taken at 30 and 60 minutes, after which the reactions were stopped by tipping in the trichloracetic acid. Aliquots of the contents of all flasks were analyzed for inorganic phosphate by the method of Gomori (12). Results are expressed as P:O ratios, i.e. μM inorganic P which disappeared per microatom oxygen consumed; the QO_2 indicates μl. oxygen uptake per flask in 60 minutes. QO_2 values from duplicate flasks prepared in all experiments did not vary more than 15%. Changes of greater than 25% in P:O ratios were considered significant. Averages of duplicate values are presented.

RESULTS

After one hour of incubation it was evident that thyroxine induced a marked acceleration of oxygen utilization by this rat heart homogenate system (Table 1). During incubation the concentration of inorganic phos-

TABLE 1. EFFECTS OF THYROXINE AND MAGNESIUM ON OXIDATION AND PHOSPHORYLATION

	QO_2	P:O	QO_2	P:O	QO_2	P:O	QO_2	P:O
Magnesium	O		5×10^{-3} *M*		10^{-2} *M*		2×10^{-2} *M*	
Control	121	0.96	157	0.87	173	0.82	219	0.65
Thyroxine	232	0	198	0.39	188	0.66	215	0.64
Control	153	0.68	185	0.60	198	0.65	158	0.80
Thyroxine	266	0	239	0.16	222	0.46	137	0.83
Control	102	0.99	182	0.64	190	0.60	168	0.68
Thyroxine	253	0.01	223	0.19	194	0.59	168	0.81

phorus in the control media decreased whereas in the presence of thyroxine it did not change. Thyroxine therefore stimulated respiration but uncoupled phosphorylation and the P:O ratios fell to zero.

Since it has been observed that magnesium inhibits the action of thyroxine under other experimental conditions (13, 14), it was of interest to determine what effect magnesium and other metal ions would have on the action of thyroxine in this system. In general, the addition to the media of magnesium, calcium, magnesium plus calcium, manganese, magnesium plus manganese, or cobalt all augmented oxidation and inhibited phosphorylation. These effects are suggested on examination of all of the data from control flasks in the absence of thyroxine. Such a general conclusion, however, may not be entirely justified on comparison of results of experiments with heart homogenates prepared at different times. The results can now be considered in more detail.

In final concentrations of magnesium below 5×10^{-3} *M* there was no significant alteration in oxygen utilization or phosphorylation in the control and thyroxine systems; but magnesium at concentrations of 10^{-2} *M* or 2×10^{-2} *M* abolished the difference in oxygen uptake between the control and thyroxine systems and prevented the uncoupling of oxidative phosphorylation by thyroxine (Table 1). Calcium at 10^{-3} *M* concentration

stimulated oxygen consumption and retarded phosphorylation in the control systems, but had little effect on the thyroxine systems (Table 2). In the presence of calcium therefore, thyroxine did not appear to have any accelerating effect on respiration. Further experiments with concentrations of calcium above 10^{-3} *M* could not be undertaken because it precipitated out of the solution. In the presence of 2×10^{-2} *M* magnesium, calcium at 5×10^{-4} *M* concentration accelerated to an equal extent the oxygen uptake of both control and thyroxine systems and inhibited phosphorylation in both systems (Table 2). Calcium, therefore, protected the uncoupling action of thyroxine against reversal by magnesium.

TABLE 2. EFFECTS OF THYROXINE, MAGNESIUM AND CALCIUM ON OXIDATION AND PHOSPHORYLATION

	QO_2	P:O	QO_2	P:O	QO_2	P:O
Calcium	0		5×10^{-4} *M*		10^{-3} *M*	
Control	100	1.17	188	0.61	319	0.38
Thyroxine	282	0	296	0	313	0.07
Control	85	1.27	183	0.61	292	0.37
Thyroxine	236	0	303	0	295	0
Calcium	0		10^{-4} *M*		5×10^{-4} *M*	
Magnesium	2×10^{-2} *M*		2×10^{-2} *M*		2×10^{-2} *M*	
Control	154	0.95	188	0.85	208	0.12
Thyroxine	155	0.99	196	0.78	219	0
Control	144	0.81	185	0.60	205	0
Thyroxine	147	0.86	159	0.61	216	0
Control	113	0.98	147	0.71	161	0.08
Thyroxine	140	0.79	143	0.71	163	0.13

Manganese, like calcium, increased oxygen utilization and impaired phosphorylation and had little effect when thyroxine was present. In combination with magnesium, manganese had less action than when employed alone (Table 3). Cobalt accelerated oxygen uptake and reduced phosphorylation; it not only blocked the effect of thyroxine on respiration but actually reduced oxygen consumption in flasks containing thyroxine (Table 4). Beryllium also impeded the action of thyroxine on oxygen utilization, but did not appreciably affect phosphorylation (Table 4). Strontium did not affect significantly either respiration or phosphorylation (Table 4). Zinc at 5×10^{-4} *M* and 10^{-3} *M* concentrations completely abolished oxidation in all flasks and barium was too insoluble to employ in concentrations comparable to those of the other cations.

DISCUSSION

A homogenous preparation of mitochondria was not used in these experiments because previous experience in this laboratory indicated that

TABLE 3. EFFECTS OF THYROXINE, MAGNESIUM AND MANGANESE ON OXIDATION AND PHOSPHORYLATION

	QO_2	P:O	QO_2	P:O	QO_2	P:O	QO_2	P:O
Manganese	0		5×10^{-4} *M*		10^{-3} *M*		2×10^{-2} *M*	
Control	126	0.95	230	0.59	190	0.66		
Thyroxine	227	0	271	0	162	0		
Control	84	1.13	201	0.50	190	0.53		
Thyroxine	231	0	204	0	250	0.05		
Control	132	0.84					169	0.58
Thyroxine	199	0					182	0.16
Control	65	1.38					221	0.37
Thyroxine	208	0.06					218	0.15
Manganese	0		5×10^{-4} *M*		10^{-3} *M*			
Magnesium	2×10^{-2} *M*		2×10^{-2} *M*		2×10^{-2} *M*			
Control	196	0.56	201	0.52	222	0.44		
Thyroxine	214	0.51	240	0.41	254	0.35		
Control	147	0.70	181	0.56	167	0.54		
Thyroxine	150	0.74	180	0.54	188	0.48		

thyroxine did not consistently accelerate oxygen utilization of heart mitochondria whereas when a fresh heart homogenate was used immediately after preparation thyroxine invariably increased oxygen uptake above the control level. The supernatant fluid from the homogenate employed in these experiments could be regarded as a crude suspension of

TABLE 4. EFFECT OF THYROXINE AND OTHER CATIONS ON OXIDATION AND PHOSPHORYLATION

	QO_2	P:O	QO_2	P:O	QO_2	P:O
Cobalt	0		5×10^{-4} *M*		10^{-3} *M*	
Control	63	1.60	148	0.63	109	0.86
Thyroxine	181	0.07	97	0.13	25	0
Control	72	1.63	129	0.93	107	1.09
Thyroxine	220	0	124	0.05	89	0
Beryllium	0		5×10^{-4} *M*		10^{-3} *M*	
Control	100	1.14	83	1.44	115	1.07
Thyroxine	201	0	173	0.05	103	0.09
Control	165	0.71	189	0.58	156	0.77
Thyroxine	291	0	210	0	183	0.04
Strontium	0		5×10^{-4} *M*		10^{-3} *M*	
Control	92	1.09	90	1.22	78	1.46
Thyroxine	314	0	263	0	254	0
Control	74	1.48	72	1.56	76	1.50
Thyroxine	235	0.05	230	0	208	0

mitochondria; P:O ratios were somewhat lower than the value of 1.8 which was obtained by Maley and Plaut (15) who used heart mitochondria with succinate as substrate. In other previous experiments concerned with the mechanism of action of thyroxine *in vitro*, Martius and Hess (3) reported that thyroxine inhibited the uptake of orthophosphate by rat liver mitochondria; they did not present data on oxygen consumption. Lardy and Feldott (4) and Maley and Lardy (6) found that thyroxine inhibited both oxidation of glutamate and oxidative phosphorylation by washed rat kidney residues, but stimulated oxidation of fatty acids at high enzyme concentrations. Judah and Williams-Ashman (16) reported that thyroxine did not alter respiration or phosphorylation by rabbit or rat liver mitochondria. Hoch and Lipmann (7) were unable to demonstrate any effect on rat liver mitochondria on addition of thyroxine *in vitro;* however, when hamster liver mitochondria were employed, thyroxine consistently uncoupled oxidative phosphorylation. Concomitant with this effect thyroxine accelerated oxidation of β-hydroxybutyrate but inhibited oxidation of glutamate, succinate, α-ketoglutarate and octanoate. In the heart homogenate used in the present experiments thyroxine both stimulated respiration and depressed oxidative phosphorylation consistently. The biologic importance of this action of thyroxine and the mechanism by which it occurs cannot be defined at present. Numerous other compounds such as calcium, manganese and cobalt as illustrated in the present experiments, and also dinitrophenol, ascorbic acid, arsenate, salicylate and dicoumarol (17) can cause uncoupling of oxidative phosphorylation. Thyroxine can be attached to or incorporated into mitochondria but this phenomenon does not appear to correlate with its metabolic activity (7, 18). Recently it has been demonstrated that thyroxine causes swelling of mitochondria and it has been postulated that thyroxine influences mechanisms of transport into mitochondria (19).

The present data bear out other investigations emphasizing the importance of metal ions in reactions involving respiration and phosphorylation. It has been found that calcium stumulates oxygen uptake by increasing ATP breakdown, and that magnesium while activating ATPase permits partial coupling of the phosphate energy of oxidation (20). Lindberg and Ernster (21), using mitochondria prepared in versene, have demonstrated that the stimulation of respiration induced by hexokinase and glucose is inhibited in the presence of calcium; the addition of manganese prevents the inhibitory effect of calcium but magnesium is without effect. Cross and his coworkers (22) have reported that magnesium in concentrations of 3×10^{-3} M to 3×10^{-2} M is essential for esterification of phosphate during respiration of rabbit kidney residues. Recently Siekevitz and Potter (23) have shown that magnesium is present within rat liver mitochondria. During incubation mitochondria lose nucleotides; this is prevented and phosphorylating ability is maintained by the addition of

magnesium. Manganese has an effect similar to that of magnesium when added after preincubation of the mitochondria, but inhibits phosphorylation when added directly to the system at the beginning of incubation. It has also been reported that magnesium protects mitochondria against the destructive effect of preincubation with phosphate (24). Whether the primary action of metal ions is on the mitochondrial membrane, or whether they act as cofactors or catalysts in enzymic reactions has not been established.

The mechanism by which magnesium inhibits the action of thyroxine and restores phosphorylating ability is not clear at present. This effect of magnesium was reported by Kuby and his associates (13) and by Bain (14), and was also observed by two other groups of workers during the period of this investigation (25, 26). It appears unlikely that magnesium combines with and removes thyroxine from the system since the concentration of magnesium necessary to restore phosphorylation in the presence of thyroxine is very much higher than the concentration of thyroxine. It is more likely that magnesium prevents the swelling of mitochondria induced by thyroxine (19). It has also been postulated that calcium and thyroxine act by decreasing the formation of oxalacetate which inhibits succinic dehydrogenase (27, 28) and that magnesium relieves this inhibition and therefore restores phosphorylating ability (29). The possible interrelationship between the actions of thyroxine and other metal ions such as manganese, cobalt and beryllium requires further investigation.

No definite relationship between magnesium and the general metabolic status in man has been established. Some investigators (30, 31, 32) have stated that abnormal levels of magnesium exist in the sera of patients with thyroid dysfunction, but others (33, 34, 35) have not confirmed these results. Recently it has been reported that the urinary excretion of magnesium rose markedly in two patients with myxedema after administration of triiodothyronine (36). As far as the authors are aware, there have been no studies of the effects of calcium, manganese, or cobalt on the peripheral action of the thyroid hormone in man.

SUMMARY

Studies on the effects of thyroxine on respiration and oxidative phosphorylation in a rat heart homogenate system have been extended. Thyroxine accelerated oxygen uptake and uncoupled oxidative phosphorylation. Magnesium inhibited the uncoupling effect of thyroxine while calcium exerted an action similar to that of thyroxine. Magnesium did not restore phosphorylating ability in the presence of thyroxine and calcium. Other cations—manganese, cobalt, strontium and beryllium—had less marked effects on respiration and phosphorylation.

REFERENCES

1. Barker, S. B.: *Physiol. Rev.* **31:** 205. 1951.
2. Wiswell, J. G., K. L. Zierler, M. B. Fasano and S. P. Asper, Jr.: *Bull. Johns Hopkins Hosp.* **94:** 94. 1954.
3. Martius, C. and B. Hess: *Arch. Biochem. and Biophys.* **33:** 486. 1951.
4. Lardy, H. A. and G. Feldott: *Ann. New York Acad. Sci.* **54:** 636. 1951.
5. Reid, L. C.: *Arch. Exper. Path. u. Pharmakol.* **219:** 466. 1953.
6. Maley, G. F. and H. A. Lardy: *J. Biol. Chem.* **204:** 435. 1953.
7. Hoch, F. L. and F. Lipmann: *Proc. Nat. Acad. Sci.* **40:** 909. 1954.
8. Klemperer, H. G.: *Biochem. J.* **60:** 122. 1955.
9. Gemmill, C. L.: *Am. J. Physiol.* **170:** 502, 1952.
10. Gemmill, C. L.: *Am. J. Physiol.* **172:** 286. 1953.
11. Wiswell, J. G.: *Am. J. Physiol.* **182:** 301. 1955.
12. Gomori, G.: *J. Lab. & Clin. Med.* **27:** 955. 1941.
13. Kuby, S. A., L. Noda and H. A. Lardy: *J. Biol. Chem.* **210:** 65. 1954.
14. Bain, J. A.: *J. Pharm. & Exper. Therap.* **110:** 2. 1954.
15. Maley, G. F. and G. W. E. Plaut: *J. Biol. Chem.* **205:** 297. 1953.
16. Judah, J. D. and H. G. Williams-Ashman: *Biochem. J.* **48:** 33. 1951.
17. Lehninger, A. L.: The Harvey Lectures 1953–54, Academic Press Inc. New York 1955.
18. Klemperer, H. G.: *Biochem. J.* **60:** 128. 1955.
19. Tapley, D. F.: *J. Biol. Chem.* **222:** 325. 1956.
20. Potter, V. R. and P. Siekevitz: *J. Biol. Chem.* **205:**893. 1953.
21. Lindberg, O. and L. Ernster: *Nature* **173:** 1038. 1954.
22. Cross, R. J., J. V. Taggart, G. A. Covo and D. E. Green: *J. Biol. Chem.* **177:** 655. 1949.
23. Siekevitz, P. and V. R. Potter:. *J. Biol. Chem.* **215:** 221. 1955.
24. Hunter, F. E. and L. Ford: *J. Biol. Chem.* **216:** 357. 1955.
25. Mudd, S. H., J. H. Park and F .Lipmann: *Proc. Nat. Acad. Sci.* **41:** 571. 1955.
26. Tapley, D. F. and C. Cooper: *J. Biol. Chem.* **222:** 341. 1956.
27. Estabrook, R. W., H. A. Neufeld and W. B. Mason: *Fed. Proc.* **13:** 205. 1954.
28. Clarke, E. C. and E. G. Ball: *Fed. Proc.* **14:** 193. 1955.
29. Tyler, D. B.: *J. Biol. Chem.* **216:** 395. 1955.
30. Soffer, L. J., D. A. Dantes, E. B. Grossman, H. Sobotka and M. D. Jacobs: *J. Clin. Invest.* **18:** 597. 1939.
31. Soffer, L. J., C. Cohn, E. B. Grossman, M. D. Jacobs and H. Sobotka: *J. Clin. Invest.* **20:** 429, 1941.
32. Dine, R. F. and P. H. Lavietes: *J. Clin. Invest.* **21:** 781. 1942.
33. Cope, C. L. and B. Wolff: *Biochem. J.* **36:** 413. 1942.
34. Bissell, G. W.: *Am. J. Med. Sci.* **210:** 195. 1945.
35. Silverman, S. H. and L. I. Gardner: *New Eng. J. Med.* **250:** 938. 1954.
36. Tapley, D. F.: *Bull Johns Hopkins Hosp.* **96:** 274. 1955.

GROWTH HORMONE CONTENT AND METABOLIC ACTIONS OF HUMAN PITUITARY GLANDS[1,2]

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MANY effects of growth hormone have been described on the basis of the response of rats to beef and hog pituitary hormone (1). These effects include, in addition to growth promotion, an increase in the plasma inorganic phosphate level (2), and the maintenance of cardiac and skeletal muscle glycogen in fasted hypophysectomized rats (3, 4). In contrast to the growth hormones of animal origin the effects of human growth hormone have never been studied directly. In view of this we have investigated the metabolic actions of human pituitary extracts in rats. In addition we have assayed the growth-promoting content of pituitaries obtained from individuals of different ages.

METHODS

The pituitary was removed at autopsy, cleaned of connective tissue and kept frozen until used. No attempt was made to dissect out the anterior pituitary since comparative studies were made between these pituitaries and pituitaries removed by operation in patients with cancer of the breast; during the latter operation the pituitary is removed piece by piece and also contains both anterior and posterior gland. The interval between time of death and autopsy varied from two to forty-eight hours and did not appear to affect the potency of the pituitary. Just prior to injection the gland was homogenized in 5 to 20 cc. of normal saline depending on the dilution desired, and was then refrozen in 1 cc. ampoules. Due to unforeseen circumstances several of the pituitaries were kept frozen for periods up to 8 months and were found on assay to be very potent.

The test animals were 7-day postoperative hypophysectomized Sprague-Dawley males rats weighing 110 to 130 gm. They were maintained on a diet of bread, milk, greens and dog chow. Injections were given subcutaneously once a day. The controls were hypophysectomized rats of the same group injected with normal saline. In experiments in which tissues were analyzed the animals were fasted for 18 hours after the last injections. They were then anesthetized with nembutal, and the tissues removed rapidly in the following order; gastrocnemius muscle, blood from the abdominal aorta, the heart and the adrenals. Glycogen was determined by the method of Good, Kramer and Somogyi (7), glucose by the Nelson-Somogyi method (8), plasma urea by the method of Seligson and Seligson (9), plasma inorganic phosphate by the Fiske-Subbarow method (10), and adrenal cholesterol by the method of Zak *et al.* (11).

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[2] Preliminary reports of these data have been presented at the annual meeting of the American Physiological Society, April 1956 (5), and at the 20th International Physiological Congress, July 1956 (6).

RESULTS

Metabolic and Tropic Effects:

For the first experiments, the pituitaries from two 57 year old males accidently killed by traumatic fractures of the skull were combined. These had been frozen for five months before assay. 36 mg. of tissue injected daily for five days induced a prompt gain in body weight which averaged 5.3 gm. a day (Table 1). This was accompanied by a proportional increase in

TABLE 1. TROPIC EFFECTS OF HUMAN PITUITARY EXTRACTS IN HYPOPHYSECTOMIZED RATS

Conditions	No. of animals	Daily wt. gain	Kidney wt.		Heart wt.		Testes wt.		Adrenal wt.		Adrenal cholesterol gm./100 gm. adrenal	Thymus wt., mg.
			Mg.	Mg./100 gm. b. wt.	Mg.	Mg./100 gm. b. wt.	Mg.	Mg./100 gm. b. wt.	Mg.	Mg./100 gm. b. wt.		
36 mg.[1]/da. ×5	6	5.3	1027[2] ±43	661 ±23	504 ±10	325 ±7	1342 ±61	864 ±34	17.8 ±.6	11.4 ±.1	3.51 ±.29	365 ±17
Control	10	0	786 ±16	667 ±13	423 ±16	347 ±16	871 ±75	702 ±68	12.8 ±.6	10.7 ±.7	7.80 ±.37	295 ±24

[1] Pituitary Nos. 1 and 5 obtained from two males aged 57 years accidentally killed; all pituitary tissue weights given are wet weights.
[2] Mean ± Standard deviation.

the weights of the kidneys, heart and adrenals, and by an increase in the width of the tibial epiphyseal cartilage. The presence of gonadotropic hormone was revealed by the marked increase in the weight of the testes, and the presence of adrenocorticotropic hormone by the decrease in adrenal cholesterol concentration (12). On histological examination the testes showed repair of both the follicle cells and interstitial cells, and the thyroid cell showed evidence of thyrotropic hormone stimulation.

When these animals were fasted for 18 hours after the last injection, the gastrocnemius and cardiac glycogen concentrations were significantly greater in the treated animals than in hypophysectomized control rats, and the plasma phosphate level was increased (Table 2). The fasting blood glucose and plasma urea concentrations were unchanged.

Of those metabolic actions that are attributable to growth hormone, the most sensitive was the increase in body weight (Table 3). With 64 μg. of

TABLE 2. METABOLIC EFFECTS OF HUMAN PITUITARY EXTRACTS IN HYPOPHYSECTOMIZED RATS

Conditions	No. of animals	Muscle glycogen, mg./100 gm.	Cardiac glycogen, mg./100 gm.	Plasma inorg. PO_4 mg./100 cc.	Fasting blood glucose, mg./100 cc.
36 mg.[1]/da. ×5	6	405 ±20	372 ±16	8.73 ±.10	51 ±3
Control	10	177 ±63	161 ±25	6.84 ±.38	48 ±9

[1] Pituitary Nos. 1 and 5.

TABLE 3. METABOLIC EFFECTS AT VARIOUS DOSE LEVELS

Dose,[1] mg./day ×5	No. of animals	Daily wt. gain, gm.	Muscle glycogen, mg./100 gm.	Cardiac glycogen, mg./100 gm.	Plasma inorg. PO_4 mg./100 cc.
10.2	3	4.6	342 ± 40	315 ± 17	—
6.4	4	6.1	281 ± 35	302 ± 18	8.33 ± .37
2.6	4	4.5	253 ± 25	278 ± 27	7.50 ± .09
1.3	4	4.5	190 ± 42	153 ± 23	8.04 ± .30
0.064	3	1.5	—	—	—
Control	10	0.0	177 ± 63	161 ± 25	6.84 ± .38

[1] Pituitary No. 2, obtained from a 46-year old man who died of carbon monoxide poisoning.

pituitary tissue injected daily, the only effect obtained was a gain in body weight. As the dose was increased to 6.4 mg. daily and above, the daily weight gain increased and leveled off, and the effects on cardiac and gastrocnemius glycogen and plasma phosphate appeared. (The pituitary used in this and the following experiment was obtained from a 46 year old man who died of carbon monoxide poisoning.) The growth response also appeared with this pituitary before evidence of other tropic effects (Table 4). The weight of the testes increased at the 1 mg. dose level, and histological evidence of thyroid stimulation appeared. The adrenal cholesterol concentration decreased and the adrenal weight increased with the 6 mg. dose.

Growth Hormone Content of Single Pituitaries:

With small doses of pituitary a linear relationship existed in all the pituitaries studied between the daily weight gain and the log of the amount injected. Figure 1 shows the lines obtained by plotting the values on three pituitaries; pituitary 2 from a 46-year old man who died of carbon monoxide poisoning, CA 1 from a 50-year old woman hypophysectomized for metastatic carcinoma of the breast, and pituitary 6 from a 8-month fetus who died $4\frac{1}{2}$ hours after birth. The minimal amount of tissue injected daily for four days that induced a daily weight gain of one gram was obtained from the line for each pituitary. Defining a growth hormone unit as this amount, the units per pituitary could be calculated. Table 5 summarizes the results of assays on seven pituitaries. Particularly interesting is the ob-

TABLE 4. TROPIC EFFECTS AT VARIOUS DOSE LEVELS

Dose,[1] mg./day ×5	No. of animals	Adrenal weights		Adrenal cholesterol, gm./100 gm adr.	Testes weights	
		Mg.	Mg./100 gm. b. wt.		Mg.	mg./100 gm. b. wt.
10.2	3	15.6	11.4	—	1256	913
6.4	4	16.3 ± 1.3	12.0 ± .83	4.00 ± .35	1222 ± 22	906 ± 23
2.6	4	14.2 ± .3	11.1 ± .1	—	1032 ± 56	809 ± 40
1.3	4	13.7 ± .5	11.2 ± .6	4.80 ± .19	979 ± 55	803 ± 73
0.064	3	12.8	9.7	6.79	—	—
Control	10	12.8 ± .6	10.7 ± .7	5.85 ± .37	871 ± 75	723 ± 68

[1] Pituitary No. 2.

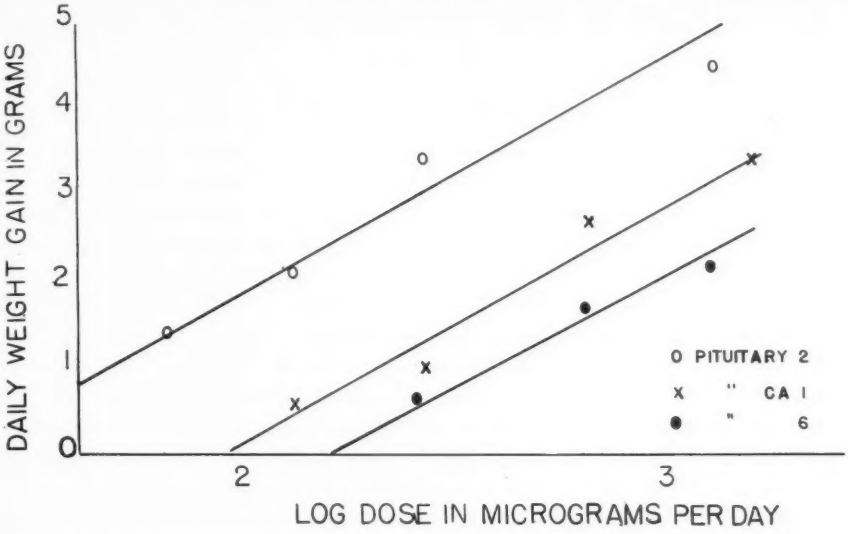

FIG. 1. Log-dose response curve of 3 human pituitary extracts in hypophysectomized rats. Pituitary 2 from a 46-year old man who died of carbon monoxide poisoning; CA 1 from a 50-year old woman hypophysectomized for metastatic carcinoma of the breast; pituitary 6 from a fetus who died four hours after birth.

servation that the pituitaries of adults 46, 65 and 72 years of age, contained at least as much growth hormone per mg. of gland or per whole gland as that of a 12-year old boy or an 8-month fetus. Included in this group are the pituitaries removed from three women hypophysectomized for metastatic cancer of the breast; these patients had been previously ovariectomized and were treated with cortisone for two days prior to operation. The hormone content of these pituitaries, as well as that of the 65-year old woman

TABLE 5. RELATION OF AGE TO GROWTH HORMONE CONTENT OF PITUITARY

Age and sex	Cause of death	Wt. of gland, mg.	Minimal[1] effective dose, μg.	Growth hormone units		
				Per mg. gland	Per whole gland	Per kg. b. wt.
72 F	Sudden death, luetic heart disease	210	70	14.5	3050	51
65 F	Metastatic cancer of stomach	126[2]	85	11.8	1003[2]	—
50 F	Hypophysectomy[3]	158[3]	178	5.6	885[3]	—
46 M	Carbon monoxide poisoning	509	42	24.0	12,200	174
38,42 F	Hypophysectomy[3]	254[3]	141	7.1	1800[3]	—
12 M	Sudden accidental death	448	155	6.5	2890	53
8 month fetus	Died 4 hours after birth	44	302	3.3	145	73
Beef	—	3053	398	2.5	7630	—

[1] Defined as the amount injected daily for four days that induced a daily wt. gain of one gm.
[2] Some tissue may have been lost in dissection.
[3] Removal at operation, so that recovery of all tissue was incomplete.

who died of metastatic cancer of the stomach, did not differ significantly from that of the glands removed from non-cancerous patients. For comparison, an adult beef pituitary was assayed (last line, tables) and was found to contain a smaller amount of hormone per mg. of gland than the human glands.

DISCUSSION

From the observation that human pituitary extracts exert the same metabolic effects in rats as beef and hog growth hormones, two inferences may be drawn: 1) that the metabolic actions of growth hormone in animals and man are similar, 2) that the ineffectiveness of beef and hog growth hormones in man (13) is due to species resistance. Instances similar to the latter exist in the cases of resistance of rats to fish growth hormone (14) and the resistance of monkeys to beef and hog growth hormones (15). It appears probable that humans would respond to human growth hormone with all the metabolic effects. Studies are in progress in our laboratory to demonstrate this.

The so-called "growth-hormone" produced by adult man is active physiologically, and retains its capacity to induce growth and to increase muscle glycogen and plasma phosphate. From the viewpoint of aging the presence of large amounts of this metabolically active hormone in the adult[3] suggests that cessation of growth and aging is not due to a deficiency of this hormone or to a change in its properties but to a change in the responsiveness of the target cells with age. An example of such a change in the response to growth hormone with age appears in the conditions of gigantism and acromegaly, due to overproduction of growth hormone in man. Before puberty the effects of the hormone are mainly on growth, resulting in gigantism without diabetes, whereas in the mature individual diabetes is usually produced along with the bony changes of acromegaly. This situation has been demonstrated experimentally in animals, in that diabetes rarely develops in kittens and puppies after injections of growth hormone in contrast to the severe diabetes that develops in the mature animals (17).

SUMMARY

Saline suspensions of human pituitary removed at autopsy produced the typical metabolic effects attributed to purified growth hormone of beef and hog origin, when injected into hypophysectomized rats. These effects included, in addition to growth promotion, the maintenance of skeletal and cardiac muscle glycogen on fasting, and an increase in the plasma inorganic phosphate level. Assays of single pituitaries revealed that approximately 100 micrograms of wet pituitary tissue contained one growth-promoting unit (the daily dose that induces a daily weight increment of

[3] It is likely that this hormone is secreted and not stored, since the growth hormone content of plasma does not change with age (16).

one gram in hypophysectomized rats). The growth hormone content of pituitaries from adults over 45 years of age did not differ significantly from that of a 12-year old boy or an eight month fetus, and the pituitaries removed from these older adults were metabolically active when injected into rats. These observations suggest, a) that cessation of growth in a man is not due to a deficiency of growth hormone, but to a change in the responsiveness of the target cells, and b) that the actions of endogenous growth hormones in animals and man are similar.

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Addendum

Since this manuscript was completed two groups of investigators have extracted a purified growth hormone from human pituitaries which is active in increasing tibial epiphyseal-cartilage width in rats (18, 19).

REFERENCES

1. Li, C. H.: The Harvey Lectures 1950–51, p. 181, Charles C Thomas, Springfield Ill.
2. Li, C. H., I. Geschwind and H. M. Evans: *Endocrinology* **44:** 67. 1949.
3. Adrouny, G. A. and J. A. Russell: *Endocrinology* **59:** 241. 1956.
4. Illingworth, B. A. and J. A. Russell: *Endocrinology* **48:** 423. 1951.
5. Gershberg, H., M. S. Druskin and M. Agulnek: *Feder. Proc.* **15:** 72. 1956.
6. Gershberg, H.: 20th International Physiological Congress, Abstracts, p. 338, July 1956.
7. Good, C. A., K. Kramer and M. Somogyi: *J. Biol. Chem.* **100:** 485. 1933.
8. Somogyi, M.: *J. Biol. Chem.* **195:** 19. 1952.
9. Seligson, D. and H. Seligson: *J. Lab. Clin. Med.* **38:** 324. 1951.
10. Fiske, C. H. and Y. Subbarow: *J. Biol. Chem.* **66:** 275. 1925.
11. Zak, B., R. C. Dickenman, E. G. White, H. Burnett and P. J. Cherney: *Amer. Journ. of Clin. Path.* **24:** 1307. 1954.
12. Long, C. N. H.: *Rec. Prog. Hormone Research* **1:** 99. 1947.
13. Shorr, E., A. C. Carter, R. W. Smith, Jr., B. J. Kennedy, R. J. Havel, T. N. Roberts, L. L. Sonkin and E. J. Livingstone: The Hypophyseal Growth Hormone, Nature and Actions, R. W. Smith, Jr., O. H. Gaebler, C. N. H. Long, Eds. (Blakiston, N.Y. 1955), p. 522.
14. Pickford, G. E.: *Endocrinology* **55:** 274. 1954.
15. Knobil, E. and R. O. Greep: *Feder. Proc.* **15:** 111. 1956.
16. Segaloff, A., E. L. Komrad, A. Flores, A. Segaloff and M. Hardestry: *Endocrinology* **57:** 527. 1955.
17. Young, F. G.: *Rec. Prog. Hormone Research* **8:** 471. 1953.
18. Gemzell, C. A. and F. Heijkenskjold: *Endocrinology* **59:** 681. 1956.
19. Li, C. H. and H. Papkoff: *Science* **124:** 1293. 1956.

A COMPARISON OF THE PITUITARY-INHIBITING, ANABOLIC AND ANDROGENIC EFFECTS OF NORETHANDROLONE IN THE PARABIOTIC RAT[1]

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THE anabolic effect of the androgens has at times been the sought-for clinical feature with the associated androgenic activity being tolerated as an undesirable side effect. Numerous attempts have been made to divorce androgenicity from anabolic activity through the preparation of new steroidal analogues of testosterone. In the past, the purported promise of all such protein-building compounds has been shattered by the demonstration of significant clinical androgenicity. A new compound, norethandrolone, was recently developed which indicated, in preliminary investigations, a preponderance of anabolic potential with little androgenic effect. This synthetic steroid has a 17-α ethyl group substituted on the 19-nor-testosterone configuration.

In the present study, the parabiotic rat technique was employed to evaluate the properties of norethandrolone since this experimental procedure offered the unique opportunity of measuring the effect of the steroid upon pituitary gonadotrophic activity simultaneously with the determination of its relative anabolic and androgenic activities. Gonadotrophin-inhibiting effects were studied by administration of norethandrolone to the castrated male rats joined in parabiosis to intact, immature female littermates. Gonadotrophin-inhibition was measured in two ways: By noting (1) the presence or absence of physiologic premature stimulation of the ovaries of the intact littermate female and (2) the morphologic alterations in the anterior lobe of the castrate male's pituitary gland. The extent of ovarian hypertrophy occurring in the intact female joined to an untreated castrate male is a quantitative measure of the extent of gonadotrophin hypersecretion from the castrate rat's pituitary gland (4). The effectiveness of the injected steroid in inhibiting gonadotrophic hormone hypersecretion can be measured by the degree of inhibition of ovarian hypertrophy in the intact littermate female.

In addition, castrates of either sex will demonstrate hypertrophy of the anterior pituitary along with histologic changes consisting of an increase in size, number and alteration in the appearance of the basophilic elements (5). These histologic changes are accompanied by an increased secretion of gonadotrophin. The morphologic demonstration of castration hyperactivity may be reversed or prevented by the administration of certain exogenous steroids in adequate dosage (6).

The relative anabolic versus androgenic potential of norethandrolone could be com-

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pared by noting the differential effect of the steroid upon the weights of the levator ani muscles and the seminal vesicles in the injected castrate male partner. While increased seminal vesicle weight classically reflects androgenic activity, the levator ani was selected as a measure of protein-anabolic effect on the basis of the demonstration by Eisenberg, Gordan and Elliot (1) and Eisenberg and Gordan (2) that the increase in size of this muscle is independent of the androgenic properties of the steroid tested. Albright (3) has demonstrated that the chemical alterations seen in growth (i.e., nitrogen, phosphorous and potassium retention) induced by testosterone propionate is indicative of actual tissue production rather than of an increase in extracellular fluid.

The following studies then are presented to offer evidence that, in the doses administered, norethandrolone can achieve protein anabolism and definite inhibition of pituitary gonadotrophin with a minimal expression of androgenicity in the rat.

MATERIALS AND METHODS

Thirty-one to thirty-three day old rats of the Long-Evans strain, weighing between 70 to 90 gm., were placed in parabiosis, employing the technique of Bunster and Meyer (7) as modified by Hertz and Meyer (8). At the time of union, castration was performed on the male right partner. The female left partner remained intact. Four groups, consisting of 5–9 pairs per group, were studied. The control group received no injections. The castrated males of the other three groups received subcutaneous injections of norethandrolone in 0.1 cc of sesame oil in daily doses of 10, 20 and 40 μg. of the steroid for 10 days. On the 11th day the animals were autopsied, following ether asphyxiation, and the pertinent endocrine glands, together with the seminal vesicles, the levator ani and gastrocnemius muscles were weighed to the nearest milligram. The tissues were preserved in Bouin's solution and prepared for section.

In order to evaluate the influence of parabiosis itself upon the experimental design, immature single male rats of the same age, weight, and strain as those placed in parabiosis were castrated and separated into two groups: One group received no injections; the other group received 40 μg. of norethandrolone daily for 10 days. On the 11th day, these animals were autopsied and studied as were the parabionts.

RESULTS

The data obtained from the parabionts are summarized in Table 1 and Figure 1. The ovaries of the female partners of the untreated pairs were markedly hypertrophied in all but one instance, as evidenced by a significant increase in ovarian weight over that noted for the intact single female of comparable age. In addition, all of the animals with ovarian enlargement showed extensive luteinization. When 40 μg. of norethandrolone were injected daily into the castrate male partners, the ovarian weights of the female partner showed no evidence of stimulation with one exception. In this latter instance, the increase in ovarian weight was minimal despite the presence of luteinization. At the lower daily dosage levels of 10 and 20 μg., an absence of ovarian hypertrophy was observed in 70% of the injected pairs. Increase in uterine size was noted in all females with stimulated ovaries as well as in some of those whose ovaries were not significantly hypertrophied. The uterine weight cannot be taken as a reliable quantitative method for determining the extent of gonadotrophin secretion inasmuch as uterine weights will reach a maximum after minimal ovarian

TABLE 1. EFFECT OF NORETHANDROLONE UPON MALE AND FEMALE PARTNERS OF PARABIOTIC PAIRS

Group	Controls		10 gamma daily		20 gamma daily		40 gamma daily	
No. of pair	7		6		5		10	
	Mean	Std. dev.*	Mean	Std. dev.	Mean	Std. dev.	Mean	Std. dev.
Female Partners:								
Ovaries	98.3	±33.4	19.7	±7.0	22.9	±8.8	16.9	±5.8
Qualitative	6 CL		0 CL		2 CL		1 CL	
Ovary response	1 Foll		6 Foll		3 Foll		9 Foll	
Uterus	122.3	±24.3	64.6	±61.6	84.7	±62.8	36.7	±11.2
Adrenals	40.1	±10.4	30.0	±3.2	25.3	±4.3	28.6	±6.6
Thymus	77.4	±35.5	117.2	±55.2	113.8	±43.1	165.4	±115.9
Thyroid	7.8	±2.3	5.7	±1.8	8.3	±2.4	7.3	±0.9
Pituitary	3.8	±1.2	2.7	±0.4	2.9	±1.2	2.8 (9)	±0.9
Rt. Gastroc.	459.4 (2)	—	371.6	—	404.0 (4)	—	423.6 (2)	—
Lt. Gastroc.	506.8 (2)	—	404.6	—	392.3 (4)	—	430.8 (2)	—
Avg. Gastroc.	483.1 (2)	—	387.6	—	398.2 (4)	—	427.2 (2)	—
Male Partners:								
Pituitary	3.8	±0.6	2.8	±0.5	2.9	±0.6	3.2	±0.9
Seminal Vesicles	9.0	±2.3	10.5	±3.4	10.0	±1.5	10.2	±2.0
Levator ani	35.2	±12.2	38.2 (5)	±9.7	43.2	±11.7	62.5	±19.3
Adrenals	29.8	±4.9	30.3	±5.2	23.7	±5.7	24.4	±1.4
Thymus	118.6	±66.3	70.7	±41.6	110.1	±13.3	117.8	±52.2
Thyroid	7.3	±1.3	7.8	±1.5	10.0	±3.0	7.0	±1.4
Rt. Gastroc.	428.4 (2)	—	400.1	—	423.0 (4)	—	453.1 (2)	—
Lt. Gastroc.	431.5 (2)	—	396.9	—	428.0 (4)	—	431.7 (2)	—
Avg. Gastroc.	430.0 (2)	—	398.5	—	425.5 (4)	—	442.4 (2)	—

* Std. Dev. =Standard Deviation.
Note: All weights expressed as mean mgs. for number of rats in group. Number in parentheses indicates number of pairs included.
CL =corpus luteum.
Foll =follicle.

stimulation. The lower doses of norethandrolone did not produce complete pituitary suppression. This was manifested by uterine stimulation with little or no evidence of ovarian stimulation.

The pituitary glands of the castrated males of the untreated pairs showed significant hypertrophy when compared with the pituitary glands

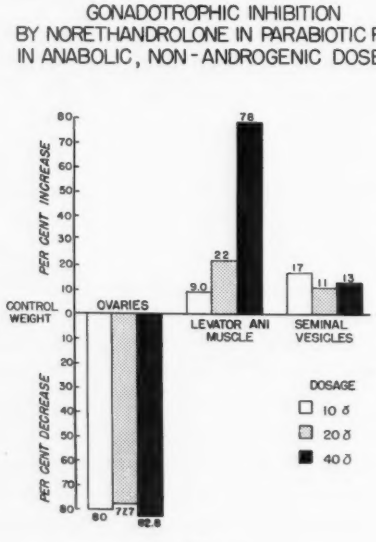

Fig. 1

TABLE 2. EFFECT OF NORETHANDROLONE UPON SINGLE MALE CASTRATE RATS

	Controls	40 gamma daily	P value*
No. of rats	9	10	
Seminal vesicles	8.6	12.7	less than .01
Levator ani	19.7	42.8	less than .01
Adrenals	24.4	25.1	greater than 0.3
Thymus	318.6	238.4	—
Thyroid	7.8	8.8	greater than 0.3
Pituitary	4.5	3.5	greater than .01
Right Gastrocnemius	596.2	603.5	greater than 0.3
Left Gastrocnemius	566.0	589.7	greater than 0.3
Average Gastrocnemius	581.1	596.6	greater than 0.3
Weight Gain	35.0	38.5	greater than 0.3

* Less than .01 = highly significant. Greater than 0.3 = not significant.

of the treated animals. The histologic changes typical of castration previously cited were prevented in those males receiving even the smallest dose level of norethandrolone. That this phenomenon was a manifestation of gonadotrophin-inhibition rather than of androgen replacement therapy is further evidenced by the lack of significant increase in seminal vesicle size at any of the three dose levels of norethandrolone. Anabolic activity without evident androgenicity was further demonstrated by the observation of marked increase in levator ani weights at a 40 μg. dose level without evidence of seminal vesicle stimulation.

A significant increase in the adrenal weights of the female partners of the untreated pairs over those of the female partners of treated parabionts is obvious at all dose levels of norethandrolone. This may be attributed to secondary adrenal stimulation following the increase in ovarian function observed in the control females. Although the thymus glands of the stimulated females appear smaller than those whose partners received norethandrolone no significance is attached to these differences because of the extreme variability in thymic size in all animals within any one group.

Neither the weights nor the histologic appearance of the thyroid glands of any of the groups suggest any effect of norethandrolone upon this gland.

Statistical analysis was performed on the data obtained from the single male castrated rats (Table 2). The results obtained from the parabionts were confirmed with two notable exceptions. A moderate, though statistically significant increase in seminal vesicle size was obtained with 40 μg. of norethandrolone. It was also noted that the levator ani muscle responded to a lesser degree in the single animals than in the parabionts receiving comparable amounts of the steroid.

Dissections of the gastrocnemius on the single rats showed no significant change effected by norethandrolone, nor was any significant difference in total body weight of the animal achieved by this steroid.

DISCUSSION

Figure 1 graphically represents the percent change from parabiotic control organ weights of the levator ani muscles and seminal vesicles of the

castrate male and the ovaries of the contralateral intact female for all the treated pairs. Comparison of ovarian weights makes it strikingly apparent that nearly complete gonadotrophin-inhibition was achieved at even the lowest dose level of norethandrolone. Differences in the pituitary gland sizes and histologic studies of the castrated rat pituitary glands in the treated and untreated animals confirm the conclusions derived from the data of Figure 1 relevant to ovarian weights.

With the highest dose level of norethandrolone injected, complete gonadotrophin-inhibition was achieved along with a marked anabolic effect, as noted by a 78% increase in levator ani weights. An insignificant degree of androgenicity was manifested, as evidenced by failure of the seminal vesicles to show a significant increase in weight. To appreciate fully the potency of norethandrolone as an anabolic agent without concomitant androgenicity, comparison must be made with the data of Gordan *et al.* (9) in which seminal vesicle weights of experimental rats treated with 100 μg. of methyltestosterone daily over a 30-day injection period were nearly tripled in achieving levator ani weights slightly less than those produced by 40 μg. of norethandrolone in this study. Hertz and Meyer (10) demonstrated that a 10-day experimental period was adequate to reveal androgenic activity by seminal vesicle stimulation in 31 to 33-day-old castrate rats. Comparison of our results with the data presented in the study cited above emphasizes the relative potency of norethandrolone as a gonadotrophin inhibitor and anabolic agent, with only minimal androgenicity. The dose of testosterone propionate required for complete inhibition of gonadotrophic secretion was 15 μg., at which level seminal vesicle size was increased nearly five times over the control values. As noted previously, even 40 μg. of norethandrolone showed no significant increase in seminal vesicle size of the parabionts, despite a 78% increase in weight of the levator ani muscle. Moreover, one-fourth this dose of norethandrolone produced a comparable degree of gonadotrophin-inhibition, further emphasizing that the pituitary-suppressing properties are not due to any androgenic potential the drug may have.

The increase in seminal vesicle size in the single male castrates after administration of 40 μg. of norethandrolone per day, although highly significant statistically, indicates relative insignificant androgenicity when compared with other testosterone derivatives currently available. One might explain the appearance of androgenic activity in the single rats at the same dose level that failed to demonstrate it in the parabionts by the following hypotheses: (1) Since the two parabionts had nearly twice the total body mass of the single animals to which the same amount of steroid was administered, the dose per body weight was actually less in the united animals; or (2) if norethandrolone is inactivated by the liver, as are other testosterone derivatives, then the same amount of compound will be exposed to twice the amount of hepatic tissue. The smaller control levator ani

weights in the single castrate control animals cannot be adequately explained.

The hypertrophy of the pituitary glands of the control males may be expected as a result of castration and was prevented by even the smallest dose of norethandrolone administered in this study.

The data on the gastrocnemius muscle weights indicate that this muscle does not demonstrate anabolic activity with as great a degree of sensitivity as does the levator ani. The discrepancy between myotrophic potency demonstrated by the levator ani and the gastrocnemius suggests that different factors might operate in the growth of these two muscles. The parabionts of either sex in which gonadotrophic hypersecretion occurred had larger gastrocnemius muscles than their fellows receiving the smaller doses of norethandrolone. As the dose of steroid is increased, the deficit in gastrocnemius weight is reduced and even overcome. Conceivably a pituitary factor (i.e., growth hormone) might be produced by the hyperactive gland, which is inhibited, as is gonadotrophin, by norethandrolone (11). This factor may manifest its anabolic potency on the gastrocnemius, but not on the levator ani at the levels in which it is present. After pituitary inhibition is achieved, this theoretic principle may no longer be effective and anabolic activity is primarily accounted for by norethandrolone to which the levator ani is more sensitive. Further studies will be done to evaluate this possibility.

SUMMARY

Norethandrolone, a steroid synthesized from estrone with a chemical configuration similar to testosterone, demonstrated the ability to suppress gonadotrophin-hypersecretion in castrate male parabionts at doses which were anabolic without being significantly androgenic.

This gonadotrophin-inhibition was not the result of androgenic activity.

In single male castrates, less anabolic and greater androgenic activity was demonstrated for equivalent dosage levels of norethandrolone than in the parabiotic animals. This demonstrated androgenicity is probably a function of the size of the dose in relation to total body weight and implies a threshold level beyond which androgenic potential of the steroid may become overt.

The gastrocnemius muscle, unlike the levator ani muscle, did not respond to the anabolic effect of norethandrolone.

Addendum

The question of estrogenic activity of norethandrolone was raised when this paper was read before the 38th Annual Meeting of The Endocrine Society in Chicago, Illinois, June, 1956. We have administered 40 and 80 μg. per day of norethandrolone to castrate adult female rats without evidence of vaginal cornification. Administration of comparable doses to

immature 21-day-old female rats resulted in uterine enlargement. A personal communication from Dr. Richard A. Edgren of the Division of Biologic Research at G. D. Searle and Company showed that administration of 0.1 mg. of norethandrolone per day to castrate adult female rats failed to show any estrogenic activity. The progestational effect of the compound has been adequately demonstrated clinically.

On the basis of the lack of estrogenic activity and the effect the compound has upon increasing the uterine weight in rats, associated with the progestational effect in the human, this preparation must be considered as being uterotrophic and not estrogenic.

Acknowledgments

We are grateful to Miss Elizabeth Street for her assistance in the statistical analyses of our data. Appreciation is expressed to Dr. Robert Craig of the Division of Clinical Research of G. D. Searle & Co. for generous supplies of norethandrolone (Nilevar®) used in these studies.

REFERENCES

1. Eisenberg, E., G. S. Gordan and H. W. Elliott: *Endocrinology* **45:** 113. 1949.
2. Eisenberg, E. and G. S. Gordan: *J. Pharm. and Exp. Therap.* **99:** 38. 1950.
3. Albright, F.: The Harvey Lectures **38:** 123. 1942–43.
4. Meyer, R. K. and R. Hertz: *Am. J. Phys.*, referred to in Hertz, R. and R. K. Meyer: *Endocrinology* **21:** 756. 1937.
5. Finnerty, J. C., H. S. Kupperman and R. K. Meyer: *Proc. Soc. Exp. Biol. and Med.* **44:** 551. 1940.
6. Meyer, R. K. and C. Biddulph: *Am. J. Phys.* **134:** 141. 1941.
7. Bunster, E. and R. K. Meyer: *Anat. Rec.* **57:** 339. 1933.
8. Hertz, R. and R. K. Meyer: *Endocrinology* **21:** 756. 1937.
9. Gordan, G. S., E. Eisenberg, H. D. Moon and W. Sakamoto: *J. Clin. Endocrinology* **11:** 209. 1951.
10. Hertz, R. and R. K. Meyer: *Endocrinology* **21:** 756. 1937.
11. Zwecker, I. T.: *Arch. Pathology* **30:** 461. 1940.

WORK PERFORMANCE OF RATS FOLLOWING PARTIAL HYPOPHYSECTOMY

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HYPOPHYSECTOMY in the rat causes a profound muscular asthenia. In acute experiments work performance can be normalized by treatment with adrenal cortical extract (ACE) plus vasopressin (3). In the present report it is shown that removal of any part of the hypophysis causes an acute suppression of work which can be corrected by ACE only when the posterior lobe is present. For several weeks following posterior lobectomy the rats show subnormal work output which is not corrected by vasopressin. By six weeks following posterior lobectomy the work capacity of the rat has been spontaneously restored to a normal or nearly normal output.

METHODS

Male rats of the Sprague-Dawley strain were obtained from the Upjohn breeding colony and maintained on Archer Dog Pellets until they reached a weight of 200 ± 2 gm. In some experiments the rats were subjected to the stimulation of muscle immediately following operation and in others there was a recovery period of either 30 or 42 days prior to the work test. All operations were done under barbiturate anesthesia. In order to remove the posterior lobe the anterior lobe was first exposed and divided by a fine needle. The tips of fine curved forceps were inserted into this incision and the two halves of anterior lobe spread apart so that the posterior lobe could be contacted with a capillary pipet and gently removed by negative pressure. The objective of this operation was achieved in over 80% of the animals. It was almost always possible to determine success or failure at the time of operation. The work test was done according to Ingle (2).

The gastrocnemius muscle of the left hind leg was weighted with 100 gm. A Nerve Stimulator Model B. (Upjohn) was used to deliver 5 pulses per second. The duration of each pulse was 20 milliseconds and the intensity was 20 milliamperes. The distance the weight was lifted was recorded on automatic work adders. Each recorder revolution represented approximately 400 gram-centimeters of work. Stimulation was continued until death or for 48 hours. Temperature was constant at $28 \pm 0.5°$ C.

Sterile solutions were administered into the jugular vein at the rate of 20 ml. per 24 hours by means of a constant infusion machine which permitted the study of 6 rats simultaneously. The ACE (Upjohn) represented the glycogenic equivalent of 0.1 mg. per ml. of hydrocortisone (1 unit). The preparation of the vasopressin has been described (5). In most of the experiments pyrogen-free sterile solutions (Baxter) of either saline or glucose were used.

In the animals studied several weeks following operation the urine volume was measured on each of the last two days and is expressed in the tables as the average of two collections.

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EXPERIMENTS AND RESULTS

Within each experiment comparisons of various treatments and conditions were made simultaneously. The details of each experiment are given in the tables.

Experiment 1 (Table 1) involved adrenalectomized-hypophysectomized rats subjected to work immediately following the double operation. It was confirmed that ACE alone supports the capacity of the adrenalectomized-

TABLE 1. WORK PERFORMANCE OF 200 GRAM MALE RATS DURING 48 HOURS IMMEDIATELY FOLLOWING HYPOPHYSECTOMY. AVERAGES AND STANDARD ERRORS, 20 UNITS ACE FIRST 24 HOURS, 10 UNITS ACE SECOND 24 HOURS

Experiment	Operation	N rats	Treatment	Recorder revolutions
1	Adrenalectomy-hypophysectomy	15	saline only	24,149 ± 1,245
	Adrenalectomy-hypophysectomy	15	ACE only	60,047 ± 2,997
	Adrenalectomy-hypophysectomy	15	ACE plus 0.02 units vasopressin	94,040 ± 3,930
2	Sham operation	17	saline only	89,994 ± 3,475
	Hypophysectomy (complete)	17	saline only	18,293 ± 3,028
	Partial hypox (post out, ½ ant out)	18	ACE only	56,824 ± 5,994
	Partial hypox (post in, ant out) diuresis	15	ACE only	52,463 ± 4,611
	Partial hypox (post in, ant out), no diuresis	12	ACE only	89,448 ± 3,518
3	Sham operation	16	saline only	93,236 ± 2,837
	Hypophysectomy (complete)	16	saline only	18,697 ± 1,554
	Partial hypox (ant out, post in)	16	saline only	21,822 ± 2,334
	Partial hypox (ant in, post out)	16	saline only	41,328 ± 2,798
	Partial hypox (½ ant out, post out)	16	saline only	30,611 ± 3,764
	Partial hypox (½ ant out, post in)	16	saline only	46,901 ± 4,279

hypophysectomized to work much better than rats treated with saline only and that ACE plus vasopressin normalizes the work performance under these conditions.

In Experiments 2 and 3 (Table 1) it is shown that removal of any part of the hypophysis suppresses work output. When the entire anterior lobe is removed the presence of the posterior lobe does not support work. However, when the remaining posterior lobe is functional, as shown by absence of diuresis, work performance can be normalized by treatment with ACE. When the animal exhibits diuresis, despite the presence of posterior lobe, the work output remains subnormal and it is assumed that the posterior lobe is damaged and is incapable of releasing vasopressin. The presence of one-half of the anterior lobe even in the absence of the posterior lobe permits the partially hypophysectomized rat to do more work than does the completely hypophysectomized rat.

TABLE 2. WORK PERFORMANCE OF PARTIALLY HYPOPHYSECTOMIZED RATS KEPT FOR 30 DAYS FOLLOWING OPERATION AND GIVEN SALINE ONLY DURING WORK. AVERAGES AND STANDARD ERRORS

Experiment	Operation	N rats	Gm. final wt.	Cc. 24 hour urine	Total average recorder revolutions
4	Sham operation	16	320 ± 9.6	14.6 ± 1.8	94,572 ± 4,263
	Hypophysectomy, complete	15	157 ± 2.5		3,845 ± 651
	Partial hypox; ant in, post out	15	296 ± 4.5	27.9 ± 2.0	72,328 ± 4,543
	Partial hypox; ½ ant in, post out	21	303 ± 7.2	25.8 ± 2.3	54,800 ± 3,302
	Partial hypox; ½ ant in, post in	16	324 ± 7.1	13.4 ± 1.8	81,141 ± 3,485

TABLE 3. WORK PERFORMANCE OF POSTERIOR-LOBECTOMIZED RATS KEPT FOR 30–42 DAYS FOLLOWING OPERATION. AVERAGES AND STANDARD ERRORS

Experiment	Operation	N rats	Days recovery	Gm. final wt.	Cc. 24 hour urine	Treatment during work	Total average recorder revolutions
5	Posterior-lobectomy	16	30	314± 5.0	26.4±1.6	saline only	68,347±2,915
	Posterior-lobectomy	16	30	318± 4.8	28.2±2.6	saline plus 0.02 u. vasopressin	65,634±3,363
6	Sham-operated	15	42	340± 4.9	11.7±0.9	saline only	89,676±4,952
	Posterior-lobectomy	9	42	309± 7.8	19.6±2.7	saline only	86,519±7,994
	Posterior-lobectomy	9	42	302±12.9	19.9±2.2	saline plus 0.02 u. vasopressin	79,994±6,547
	Posterior-lobectomy	15	42	328± 8.1	31.0±2.7	5% glucose only	89,074±2,471
	Posterior-lobectomy	14	42	323± 8.1	34.0±2.1	5% glucose plus 0.04 u. vasopressin	90,270±4,241

In Experiments 4 (Table 2), 5, and 6 (Table 3), the partially hypophysectomized rats kept for 30 and for 42 days following operation were found to work less than sham-operated rats. When one-half of the anterior lobe has been removed the presence of the posterior lobe permits better recovery of vigor than in its absence. Treatment of posterior-lobectomized rats with vasopressin fails to improve work performance. There is an established tendency of these animals to improve with time so that work performance is essentially normal by six weeks following partial hypophysectomy. The diuresis which appears immediately after posterior-lobectomy abates with time and never remains severe.

DISCUSSION

In an earlier study (3) it was shown that vasopressin has a marked extra-adrenal effect upon the work of the hypophysectomized rat when given with ACE in acute experiments. This is confirmed in the present study. It is also shown that ACE can correct the work deficit of the anterior lobectomized rat when the posterior lobe remains present and functional. The loss of vigor which immediately follows removal of the posterior lobe is apparently due to a deficiency of vasopressin. The posterior-lobectomized rat continues to show subnormal performance until about six weeks following operation but the signs of deficiency of vasopressin (diuresis) are mild and treatment with vasopressin during this time does not improve work performance. It can be assumed that the subnormal work performance is due to a deficiency in one or more other factors, possibly of the anterior lobe which incurs some damage and circulatory derangement as the result of posterior lobectomy.

The objective of this study was to test the possibility that the posterior lobectomized rat might continue to show improved work output in response to treatment with vasopressin during several weeks following operation. This was not found to be true. There are other reports supporting the conclusion that a severe deficiency of vasopressin does not persist following removal of the posterior lobe of the hypophysis in the rat: the proximal end of the severed pituitary stalk becomes reorganized into new lobe tissue that is capable of storing neurosecretory material containing vasopressin (1); although the serum of recently hypophysectomized rats contains but

little vasopressin the serum of rats hypophysectomized for 30 days or longer contains only slightly less than normal amounts of vasopressin (4).

SUMMARY

In this study of the ability of rats to do muscle work in response to faradic stimulation following partial hypophysectomy the following points were established:

In adrenalectomized-hypophysectomized rats subjected to the work test immediately following operation it was confirmed that work performance can be normalized by treatment with adrenal cortical extract plus vasopressin.

Also in acute experiments it was shown that adrenal cortical extract can correct the work deficit of the anterior lobectomized rat when the posterior lobe remains present and functional.

The work output of rats having one-half an anterior lobe remaining is much greater than that of completely hypophysectomized rats and is still greater, although subnormal, when the posterior lobe and one-half the anterior lobe remain *in situ*. This is true immediately following operation and at 30 days post-operatively.

The work performance of the posterior lobectomized rat is subnormal at 30 days following operation and is not improved by treatment with vasopressin. Vasopressin also failed to improve work performance at 42 days following removal of the posterior lobe; two groups of rats regained normal vigor by that time. Although severe diuresis was noted in all posterior lobectomized rats for the first few days following operation, it became mild in all animals by 30 and 42 days post-operatively.

Acknowledgment

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REFERENCES

1. Billenstien, D. C. and T. F. Leveque: *Endocrinology* **56:** 704. 1955.
2. Ingle, D. J.: *Endocrinology* **34:** 191. 1944.
3. Ingle, D. J. and C. H. Li: *Endocrinology* **57:** 383. 1955.
4. Lloyd, C. W. and S. Pierog: *Endocrinology* **56:** 718. 1955.
5. Taylor, S. P., Jr. and V. du Vigneaud: *J. Biol. Chem.* **205:** 45. 1953.

HORMONAL INDUCTION OF MELANOGENESIS IN HYPOPHYSECTOMIZED KILLIFISH (*FUNDULUS HETEROCLITUS*)[1]

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THE experiments reported in this investigation were instigated by the accidental discovery, described below (Series XXI), that chronic treatment with prolactin stimulated melanogenesis in hypophysectomized killifish, *Fundulus heteroclitus* (Linn.).

In discussing the complex problems involved, a distinction must be made between 1) physiological regulation of the state of concentration or dispersion of the melanophores, and 2) morphological processes of depigmentation, melanization, and new pigment cell formation. It has been repeatedly pointed out that the two phenomena appear to be interrelated (1, 2). Conditions which favor melanophore dispersion (illumination against a dark background, blinding) promote increased pigmentation and the development of new pigment cells. Conditions which favor aggregation of the melanin granules (illumination against a light background) retard or inhibit the development of melanophores and lead to a partial loss of pigment from cells already present. This is to be expected since the afferent pathways, through the photoreceptors, are the same. It does not necessarily follow that the effector pathways are identical.

The physiological regulation of color changes in Fundulus, reviewed by Parker (2), appears to depend primarily on nervous control. It is well known that the melanophores of this species are unresponsive to the melanophore-dispersing hormone, intermedin. A latent capacity to respond is revealed, however, by denervation, and, in fact, the use of Fundulus with faded denervated caudal bands has been recommended by Abramowitz (3) for the assay of intermedin. On the other hand, the melanophores of Fundulus are extremely sensitive to the melanophore-concentrating hormone of the teleostean pituitary. The literature on this subject has been reviewed by Pickford and Atz (4).

The nervous regulation of the melanophores permits rapid color changes even in hypophysectomized fishes, but the presence of the pituitary is necessary for normal maintenance of background adaptations. This was clearly demonstrated by Healey (5, 6) in his studies of the European minnow, *Phoxinus phoxinus*. It is well known, moreover, that removal of the pitui-

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tary in various teleostean species leads to a partial loss of melanin from the melanophores. Fundulus is no exception. Despite contrary observations reported by Matthews (7), the senior author has repeatedly observed that hypophysectomized Fundulus become markedly paler than the controls. The role of the pituitary in promoting the formation of new pigment cells was studied by Osborn (8, 9) on the summer flounder (*Paralichthys dentatus*) and the catfish (*Ameiurus nebulosus*). Osborn showed that the formation of new melanophores on the normally pale underside could be induced by illumination from below, provided the fish had been blinded. The work of Veil (10, 11) on *Cyprinus carpio* showed that blinding leads to an increase of melanophore-dispersing hormone in the blood. Osborn found that removal of the pituitary abolished the response to ventral illumination, in agreement with the work of Abramowitz (12) who showed that the blood of hypophysectomized Fundulus does not contain the melanophore-dispersing hormone. Although these experiments suggested very strongly that intermedin was concerned in the process of new pigment cell formation, Osborn did not confirm this hypothesis by chronic administration of this or other pituitary hormones. In experiments on the bitterling, *Rhodeus amarus*, Brantner (13) found that mammalian anterior lobe extracts, added to the aquarium water, caused a marked increase in the abundance of melanophores. Posterior lobe extracts had a similar, but less striking, effect. Although the recipients were not hypophysectomized, this experiment would support the hypothesis that some pituitary anterior lobe hormone might be concerned in the process of melanophore multiplication. The response to either preparation was enhanced when the fish were kept on a black background, which maintains a natural state of maximal melanophore-dispersion.

A number of investigators have found that injections of pituitary preparations induced melanization in red or yellow goldfish. Ito (14) observed this response after treatment with an anterior lobe extract, whereas a posterior lobe preparation had no such effect. Chavin (15), who employed hypophysectomized recipients, found that melanin appeared in three to four days after an injection of ACTH, whereas cortisone and intermedin were ineffective. Similar results could be elicited in tissue culture (16), and it was concluded that ACTH had a direct action, not mediated through the adrenal cortical tissue. The situation is clearly different in Fundulus. Unpublished experiments, confirmed in the present investigation, showed that prolonged treatment with ACTH failed to restore the depleted pigmentation of hypophysectomized recipients.

Investigations of the role of the thyroid in teleostean pigmentation, reviewed by Pickford and Atz (4), indicate that in many species prolonged treatment with thyroxine or thyrotropin leads to a depigmentation of the melanophores. This is true, for example, in the goldfish (17). However, in experiments at the Bingham Oceanographic Laboratory, we have not ob-

served any significant effects on the pigmentation of hypophysectomized Fundulus following thyroid feeding, injections of thyroxine or prolonged treatment with low doses of thyrotropin (18). Previous experiments have also shown that the growth hormone has no significant effect on melanin pigmentation (19, 20). Participation of the sex hormones, at least in Fundulus, has been excluded by experiments with gonadotropins, reported briefly below, and androgens (Pickford, unpublished), although either treatment stimulates the development of xanthophores.

In the Amphibia, intermedin elicits both melanophore dispersion and probably also melanogenesis (21). However, Chavin (22) reported that intermedin had no effect on melanophore differentiation in salamander larvae. At this stage the larval melanocytes may not have acquired competence to respond to endocrine influence. Dalton and Krasner (23) obtained negative results with amphibian larvae immersed in ACTH solutions. Prolonged treatment with thyroxine apparently causes depigmentation in axolotls, as in goldfish (24). The investigations of Blair (25), on the other hand, brought to light the interesting fact that prolactin caused a significant increase in the number of melanophores in the throat region of juvenile toads, *Bufo fowleri*, without inducing melanophore dispersion. Androgens, and chorionic gonadotropin, had a more striking effect but also induced melanophore dispersion. Since the recipients were not hypophysectomized, secondary reactions mediated through the pituitary cannot be excluded. Nevertheless, the findings implicate a possible role of prolactin in amphibian melanogenesis.

The problem of melanogenesis in higher vertebrates has been reviewed in a series of articles by Lerner and his collaborators (26, 27, 28). There is a general agreement that intermedin participates, and probably plays a primary role in melanization, although other factors are operative. The possible role of prolactin has not been studied.

METHODS

Fish varying in length from 53 to 77 mm. were employed, but the majority were two-year olds, about 60 mm. in length. Following capture, in the vicinity of New Haven, Conn., they were kept for at least 6 weeks in the laboratory in salt water so as to become fully adjusted to aquarium life, and to permit elimination of diseased individuals. The pituitary was then removed by the method described previously (29), and at least another two months was allowed to elapse in order that the target organs of the pituitary might pass into a state of complete regression. Experimental conditions were similar to those used in previous experiments (18, 19, 20, 29). Hypophysectomized Fundulus cannot survive in fresh water (30). The temperature was regulated at 20° C, and, unless otherwise stated, illumination was for 8 hours per day with 15 watt white fluorescent lamps standing ca. 8 inches above the water level. The fish were fed daily with Aronson's mixture containing a trace of iodine, as recommended previously (29), and a supplement of frozen daphnia or brine shrimp was given approximately three times a week. Once a week, as a rule, the fish were given a dip in dilute malachite green solution (1:15,000 in fresh water, for 30 seconds or 1 minute) as a prophylactic against skin infections. When

symptoms of a more serious nature were noted, individual fish were sometimes isolated for a few days and treated with aureomycin (soluble Aureomycin, citric acid with sugar, Lederle Laboratories; 50 μg. per gallon) in the aquarium water. This treatment was avoided, except in rare instances, during the actual period of the experiment.

The hormones were dissolved in 0.6% NaCl and the volume injected was held constant (0.005 ml. per 0.5 gm. weight).

Injections, and visual observations of the degree of darkening and appearance of new melanophores, were made under anesthesia with tricaine methane sulfonate. This anesthetic causes the melanophores to pass into a state of maximal dispersion.

Autopsies were made on the day following the last injection. Weight and length were compared with initial measurements at the start of the experiment. The weight of the gonad, expressed as a gonosomatic index (Wt. of gonad × 100/Body wt.), served as a measure of gonad stimulation. Mean thyroid cell height was determined as previously (18) on sections from Bouin fixed material. The adrenal cortical tissue was studied on sections of the right head kidney, fixed in Zenker's fluid.

Preliminary studies of the origin of new melanophores were made on whole mounts of scale pockets from the ventral region. The skin was fixed in Bouin's fluid, which decalcifies the scales, washed in repeated changes of 70% alcohol containing a few drops of saturated aqueous Li_2CO_3 to remove the picric acid, and treated as follows. Decalcified scales, with the adherent epithelium and connective tissues of the scale pocket, were removed with forceps and stained deeply in a small volume of 1% aqueous benzoazurine. They were then dehydrated through acid alcohols, 70%, 95% and 100%, each containing ca. 1% HCl, and cleared in clove oil. The addition of acid is necessary to prevent the color from changing to red, and a glass needle is recommended for transfer. This method is valuable because benzoazurine stains not only the cells and their nuclei, but also the finer cell processes and connective tissue fibrillae.

Melanin assays were made by the junior author by the turbidometric method of Foster, Cook and Stamas (31), using the tails of the fish. Pretreatment of the tissue required decalcification in 70% alcohol containing 3% nitric acid, to minimize extraneous insoluble material in the homogenate. The method of fixation was different in the two series that were successfully studied (Series XXVI and XXVIII, *q.v.*) but in all cases 1 ml. of the solvent (5.6 gm. KOH+1 gm. sodium thioglycollate in 100 ml. water) was added per milligram dry weight of tissue. The mixture was heated in a water bath and allowed to cool before measurement, and the results are expressed in Klett units.

Through the courtesy of Dr. Aaron B. Lerner, intermedin assays of the prolactin preparations were made by his research assistant, Dr. M. Ruth Wright, using the method of Shizume, Lerner & Fitzpatrick (32). One MSH unit is the frog melanophore-dispersing activity of 0.04 μg. of a dried aqueous extract of beef posterior lobe powder.

EXPERIMENTS

Series XXI

Protocol. This experiment was part of a study of the response of hypophysectomized Fundulus to mammalian gonadotropins. Males, captured in May, 1954, were hypophysectomized between Sept. 21–23 and injections were begun on Nov. 15, approximately two months later. Groups of 5 or 6 fish each received injections of Parke-Davis Antuitrin S (4 I.U./gm. wt.), FSH (C.H.Li, 10 μg./gm. wt.), LH (Armour Lot 227–80, 1.0, 0.5, 0.1, 0.05 and 0.01 μg./gm. wt.), and prolactin (Schering Hyex 4, 10 μg./gm. wt.) thrice weekly for one month (13 injections).

Results. The gonadotropins, especially LH, stimulated the testes and caused an assumption of nuptial coloration. These results, which have been

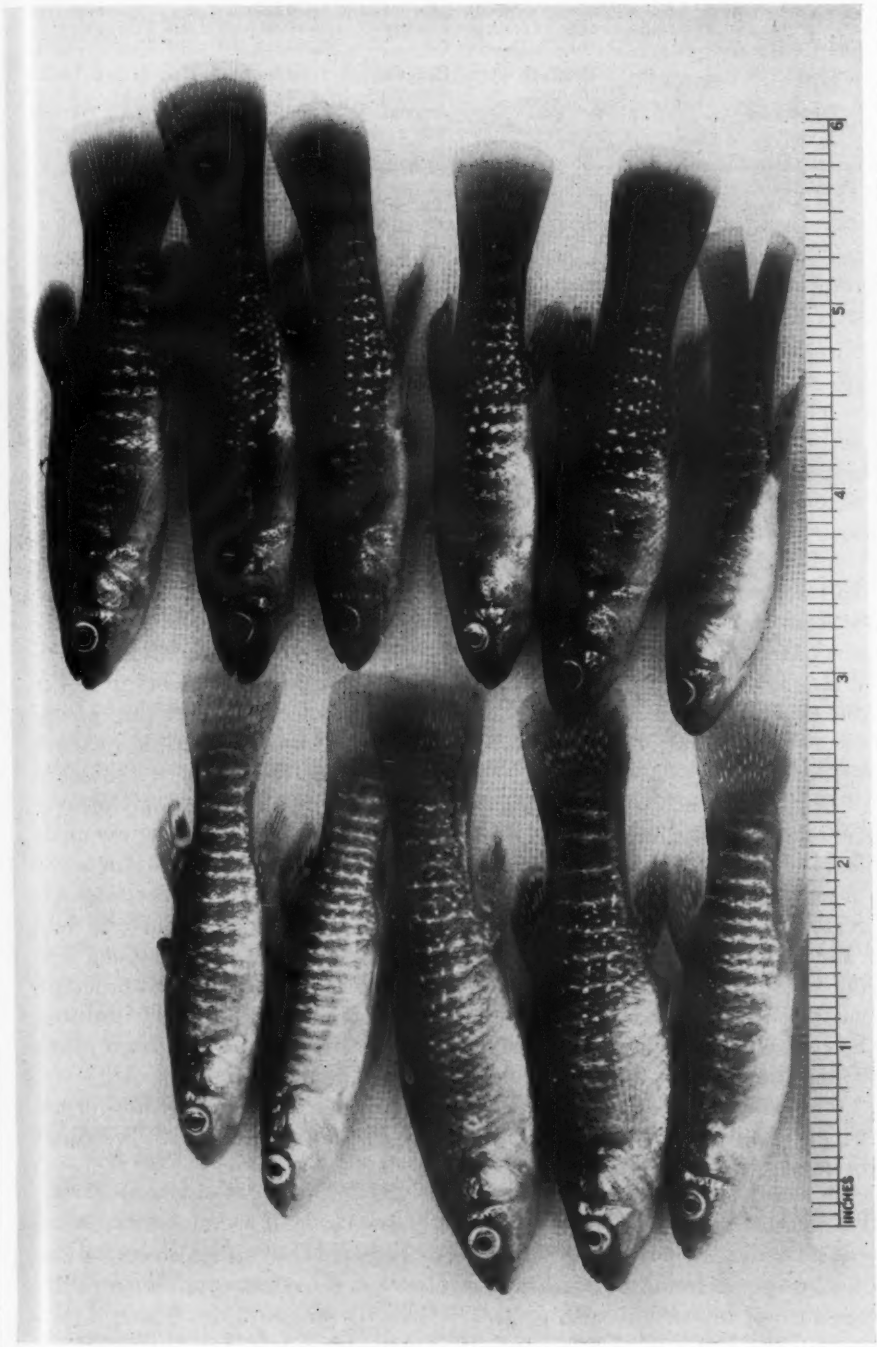


Fig. 1. Melanogenesis in hypophysectomized Fundulus after treatment for one month with Schering prolactin (Series XXI); controls on left

TABLE 1. SERIES XXI. EFFECT OF SCHERING PROLACTIN ON WEIGHT, LENGTH TESTES, THYROIDS AND ANTERIOR INTERRENAL TISSUE OF HYPOPHYSECTOMIZED MALE FUNDULUS

Treatment	Number of fish	Weight change per cent		Length change per cent		Gonosomatic index		Thyroid cell height microns		Ant. interrenal cell layers
		Avg.	s[1]	Avg.	s	Avg.	s	Avg.	s	
Prolactin 10 μg./gm.	6	0.45	±5.55	−0.02	±0.62	0.41[2]	±0.11	3.57	±0.68	2–4
Saline controls	5	−3.57	±5.09	−0.12	±0.98	0.37	±0.14	3.81	±0.65	1–3

[1] Standard deviation for small numbers.
[2] Not significantly different from controls.

summarized by Pickford and Atz (4), will not be discussed here, but it is necessary to point out that no melanogenesis was observed.

The dorsal surface of fish receiving prolactin began to darken during the second week of the experiment and inspection, at this time, revealed the appearance of new melanophores on the sides of the belly in some instances. At the end of the experiment all fish were extremely dark (Fig. 1) and 4 out of 6 had new melanophores on the normally pale ventral surface.

A study of the target organs (Table 1) indicated that the preparation had no growth promoting action, little of no gonad stimulating activity, and no thyrotropin. It is probable that some ACTH was present, since the anterior interrenal tissue showed definite signs of stimulation (an increase in the average number of cell layers from 1–3 in controls to 2–4 in the experimental fish). An assay for intermedin, made on frog skin, showed that the preparation contained 13.75×10^4 MSH units per gram, *i.e.* each fish received 1.38 MSH units per gram weight per injection.

Correspondence with Dr. Preston L. Perlman of the Schering Corporation provided the following information. The Hyex 4 preparation was made from sheep pituitaries in 1941 and, at that time, had a prolactin assay of ca. 35 I.U. per mg. by the Lyon's pigeon crop test. No data was available regarding possible contamination with other active agents, but our data (cited above) indicate that only intermedin and ACTH need be given serious consideration. The preparation has not been retested for its prolactin assay, but Dr. William C. Grant (personal communication) has found that it is extremely active in the induction of water drive in red efts, from which it may be deduced that the prolactin content remains high (33).

The carcasses of the Series XXI fish were preserved in calcium-formol which rapidly bleached the pigmentation, so that no further studies could be made.

Series XXII F

The findings reported for Series XXI suggested that either prolactin was itself responsible for melanization, or else that the effect resulted from the presence of intermedin and (or) ACTH in the preparation. Through the courtesy of Dr. C. H. Li, we received highly purified preparations of these

hormones, essentially free from active contamination, *viz.*: alpha corticotropin (intermedin-free, prepared by the method of Li *et al.*, 34), intermedin (ACTH-free, MSH assay 1.1×10^8 units per gm.), and sheep prolactin (ACTH-free and intermedin-free, prepared by the method of Cole & Li (35): 35 I.U. per mg.).

Protocol. Male Fundulus, and one female included by mistake, captured in October, 1954, were hypophysectomized on Dec. 20–21 and injections were started on April 7, 1955, approximately four months later. Each hormone was tested at two dosage levels, 10 μg. and 1 μg./gm. wt. Conditions were the same as in Series XXI and the experiment was terminated after one month (13 injections).

Results. A study of the target organs revealed, as anticipated, that the preparations were essentially free from growth promoting, gonadotropic and thyrotropic activity. Weight changes were erratic, as usual, but all groups showed a mean weight loss. The weight loss was unusually severe in the groups receiving ACTH, amounting to 29.9% at the higher dose and 10.9% at the lower dose. Length changes were negligible, although the ACTH groups showed a small mean loss of the order of 1.25% at both doses. The testes were in complete regression in all groups. In the groups receiving prolactin and intermedin, at the higher dose, the average thyroid cell height was slightly, but not significantly, greater than that of the controls (prolactin: 4.01 ± 0.67; intermedin: 4.08 ± 0.76; saline: 3.74 ± 0.58); the other groups showed no sign of incipient thyroid stimulation. The adrenal cortical tissue was not stimulated, except in the groups receiving ACTH. Corticotropin, at 10 μg./gm. wt., caused a marked increase in the average number of cell layers which rose to 3–6, as compared with 1–4 in the controls, and there was extensive proliferation into the adjacent lymphoid tissue of the head kidney. At the lower dose, 1 μg./gm. wt., corticotropin had little histologically observable effect; only one fish out of four showed visible evidence of marked adrenal cortical stimulation.

Prolactin, at 10 μg./gm. wt., caused a marked darkening of the dorsal surface, less striking than in Series XXI, but clearly evident in comparison with the pallid controls. The lower dose had only a small effect. No new pigment cells were observed on the belly of any of the prolactin-treated fish. Intermedin, at the higher dose, also caused some visible darkening, definitely less than with the higher dose of prolactin. On the other hand, this treatment stimulated the development of new melanophores which appeared first at the sides of the belly and gradually spread over the ventral surface, following the pattern of the scale pockets (Fig. 7). At the lower dose, intermedin had very little effect. The relative abundance of new melanophores, in these two intermedin-treated groups, is summarized in Table 2. Fish receiving ACTH were pale and thin, there was no stimulation of melanogenesis.

The carcasses, preserved in 70% alcohol containing 5% acetic acid, were passed through higher alcohols and cleared in methyl salicylate. Although

TABLE 2. RELATIVE ABUNDANCE OF NEW MELANOPHORES AND TURBIDOMETRIC ASSAYS OF MELANIN IN FISH RECEIVING INTERMEDIN-CONTAINING PREPARATIONS

Series and treatment	Prolactin, μg./gm.	MSH units /gm.	Serial No. of fish	Sex	New melanophores[1]	Melanin, Klett units
XXII F						
Intermedin	—	1100	16	♂	+++	—
			22	♂	++	—
			29	♂	++	—
			43	♂	+++	—
			48	♂	+++	—
			24	♀	+++	—
Intermedin	—	110	26	♂	+	—
			30	♂	+	—
			38	♂	−	—
			40	♂	−	—
			94	♂	−	—
			106	♂	−	—
XXVI						
Intermedin	—	6000	1	♀	+	43.0
			3	♀	+	46.3
			54	♂	++	49.5
			62	♂	++	46.2
			69	♂	+++	64.5
			71	♂	++	59.5
Intermedin	—	600	7	♀	+	36.8
			24	♀	−	50.5
			50	♂	+	53.7
			(53)[2]	♂	++	54.5)
Intermedin +prolactin	10	600	35	♀	+++	56.5
			36	♀	+++	70.7
			55	♂	++	56.7
			57	♂	+++	81.5
			58	♂	++	65.4
XXVIII						
Intermedin +prolactin, in light	10	600	12	♂	++	84.7
			17	♂	++	107.2
			21	♂	+++	77.4
			22	♂	+++	58.6
			32	♂	+++	84.7
The same, in dark	10	600	25	♂	++	96.0
			26	♂	++	92.2
			27	♂	+	80.0
			28	♂	+	87.6
			30	♂	+++	83.4

[1] Explanation of symbols: − = no new melanophores detected, + = a few minute new melanophores at sides of belly and in front of pelvic fins, ++ = new melanophores sparsely distributed over most of the ventral surface, +++ = new melanophores densely distributed over the whole of the ventral surface.

[2] See Table 3.

[3] This fish died 10 days before the end of the experiment.

this treatment preserved the melanin for a long time, so that the distribution of the melanophores could be studied, there was a partial, progressive fading. An attempt to determine the melanin, after 17 months, gave poor results, not concordant with visual observations and photographic records made at the time of autopsy.

Series XXVI

A consideration of the results of Series XXII F indicated that the two effects observed with Schering prolactin (Series XXI) were separable. Prolactin stimulated the formation of more melanin in the dorsal melanophores, but no new pigment cells were noted. Intermedin caused the appearance of new melanophores which spread over the ventral surface. The effective dose of intermedin, however, was high, so that similar results with the Schering prolactin could not be attributed to the action of intermedin alone. An experiment was arranged to ascertain whether a combination of intermedin, at an ineffective dose, with prolactin would simulate the response to the Schering preparation.

Protocol. Males and females, taken in May, 1955, were hypophysectomized Aug. 22–25 and injections were begun on Feb. 27, 1956, 6 months later. Experimental conditions were the same as before, except that both sexes were represented in each group. In order to obtain unequivocal evidence that no new pigment cells appeared under the influence of prolactin alone, photographs of the left side of the head and operculum were made at the beginning and end of the experiment. The preparations employed were a new sample of sheep prolactin (C.H.Li) and a new sample of intermedin (C.H.Li: MSH assay 0.6×10^9 units per gram). One group of fish received prolactin at a dose of 10 μg./gm. wt., intermedin was tested at two doses (10 μg. and 1 μg./gm. wt.), and, for the combination test, the lower dose of intermedin (1 μg./gm. wt.) was used in conjunction with prolactin (10 μg./gm. wt.).

Results. The fish did not grow during the experiment. The gonads were unstimulated except in the group receiving the higher dose of intermedin, in which the gonosomatic indices were slightly higher than would be expected for hypophysectomized subjects, as evidenced by the controls. It was not possible to establish statistical significance, on account of the small numbers since both sexes were represented. Mean gonosomatic indices for hypophysectomized males rarely exceed 0.37, and the single control male fell in this range (0.39), but the mean for the four intermedin-treated males was 0.50 ± 0.20. Similarly the gonosomatic indices of the two intermedin-treated females were 1.49 and 1.22, whereas the controls were only 0.85 and 0.51. It is therefore possible that the preparation contained a slight trace of LH, the response being of the same order as that elicited by a dose of 0.01 μg./gm. wt. of this hormone (Armour's Lot 227–80) employed in the Series XXI experiments (4). However, a small trace of gonadotropin is unlikely to have affected the results since the Series XXI experiments showed that a dose one hundred times greater had no significant effect on melanization, and the intermedin preparation employed in Series XXII F had no gonad stimulating action. The thyroids and anterior interrenals were not studied.

Prolactin alone caused a marked increase in dorsal pigmentation, but comparison of initial and final photographs (Fig. 3A, B) showed, beyond

A. INITIAL B. FINAL

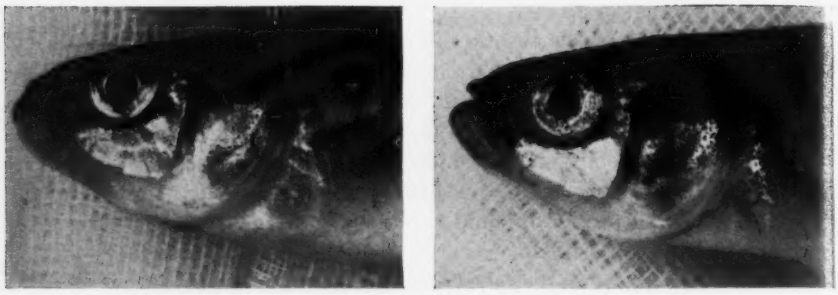


FIG. 2 INTERMEDIN 10 µg / gm

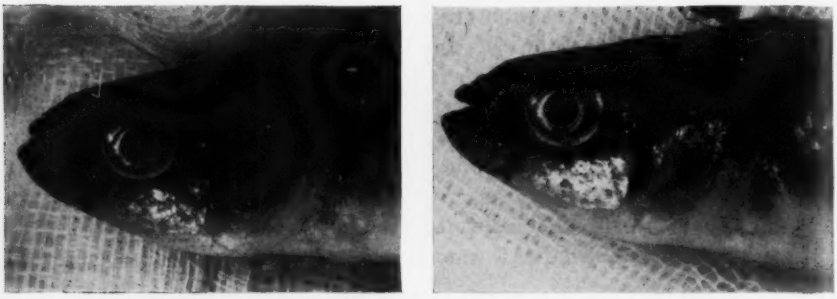


FIG. 3 PROLACTIN 10 µg / gm

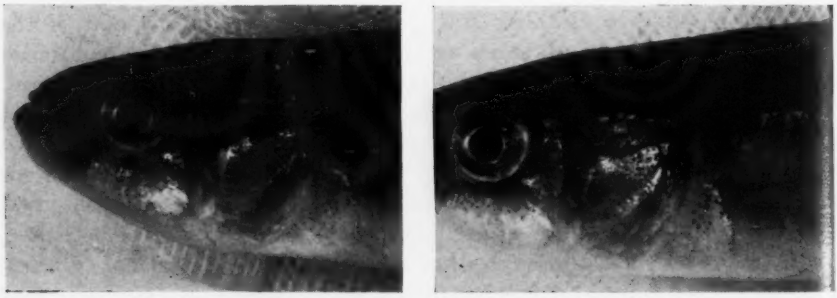


FIG. 4 SALINE

FIGS. 2–6. Left side of head and operculum of representative fish from Series XXVI: A, at the start of the experiment, B, after one month. The photographs show the appearance, or non-appearance, of new melanophores; the degree of darkening between initial and final pictures is not strictly comparable on account of differences in lighting and exposure time on the two occasions.

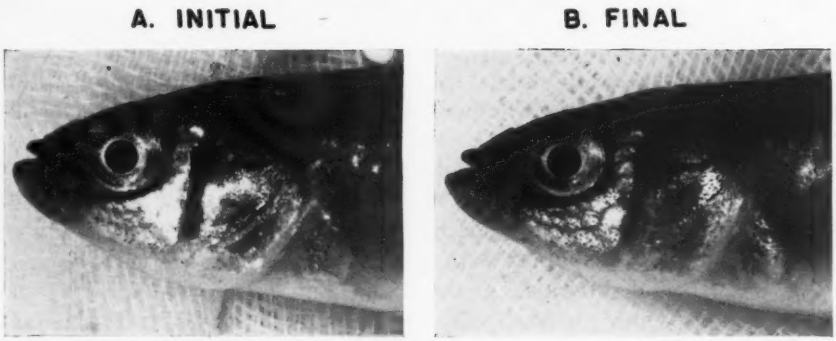


FIG. 5 INTERMEDIN 1 µg / gm

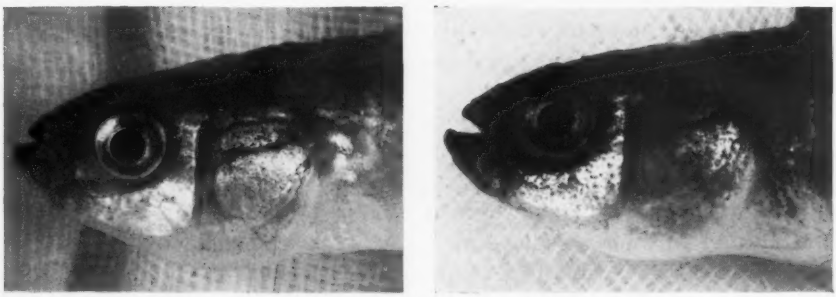


FIG. 6 INTERMEDIN 1 µg / gm PLUS PROLACTIN 10 µg / gm

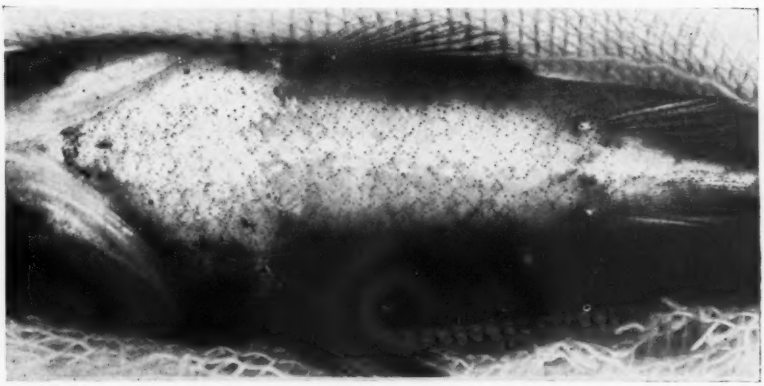


FIG. 7 INTERMEDIN 10 µg / gm

FIG. 7. The belly of a fish that had been treated for 3 weeks with intermedin (Series XXII F, 10 µg./gm.). During the final week of the experiment, subsequent to this picture, the melanophores spread over the surface so as to partially obscure the characteristic pattern of the scale pockets.

reasonable doubt, that no new pigment cells were formed, although already existing cells became more heavily pigmented. The higher dose of intermedin caused some increase in dorsal pigmentation, but this was not striking. New melanophores (Fig. 2A, B) appeared during the second and third weeks of the experiment and spread over the belly. The lower dose of intermedin had little effect; three out of four fish showed a few new pigment cells towards the end of the experiment (Fig. 5A, B). When the low dose of intermedin was combined with prolactin the results were spectacular (Fig. 6A, B); dorsal darkening was intense and was accompanied by the proliferation of new pigment cells over the throat and belly. It thus appears that a dose of intermedin which is insufficient to induce the formation of new melanophores when administered alone, can be effectively potentiated by simultaneous administration of intermedin-free prolactin. It must be noted, however, that the amount of intermedin employed in this experiment (600 MSH units/gm. wt./injection) was far greater than the known contamination of the Schering prolactin employed in Series XXI (1.38 MSH units/gm. wt./injection).

The data presented in Table 2 suggest that females may be less responsive than males, but the numbers are too small for statistical treatment.

Tails were removed from carcasses that had been fixed in acetic alcohol and cleared in methyl salicylate. They were washed in xylene, carried down through 100% and 95% alcohol, and decalcified in 70% alcohol with 3% HNO_3, after which they were washed in 70% alcohol, dehydrated through 100% alcohol, cleared in ether, and dried for the assay of melanin. It is probable that some fading may have occurred during the period of storage (9 months), but this was shorter than in the case of Series XXII F (*q.v.*) and the results appear to be significant (Tables 2 and 3). The mean value for the group receiving intermedin at 10 μg./gm. wt. was slightly, but not significantly, greater than that of the controls; at the lower dose of intermedin there was no effect. On the other hand, melanization was significantly greater in the prolactin group than in the controls ($P = <0.05, >0.02$), and the results with the combined treatment were highly significant ($P = <0.01, >0.001$). Although the assay was somewhat higher, the combined treatment did not give an increase that was significantly greater than with prolactin alone. These results suggest that when intermedin is given without prolactin, there is a continued partial failure of melanin synthesis, although new melanophores appear in abundance. Prolactin, alone or in combination, appears to promote melanin synthesis.

The melanin assays for the individual intermedin-treated fish are given in Table 2, in comparison with the relative abundance of new melanophores. Inspection of the data suggests that there is no close correlation between the two types of response.

Origin of melanophores. Preparations of belly skin were studied from a control and an intermedin-treated fish (10 μg./gm. dose). Young melano-

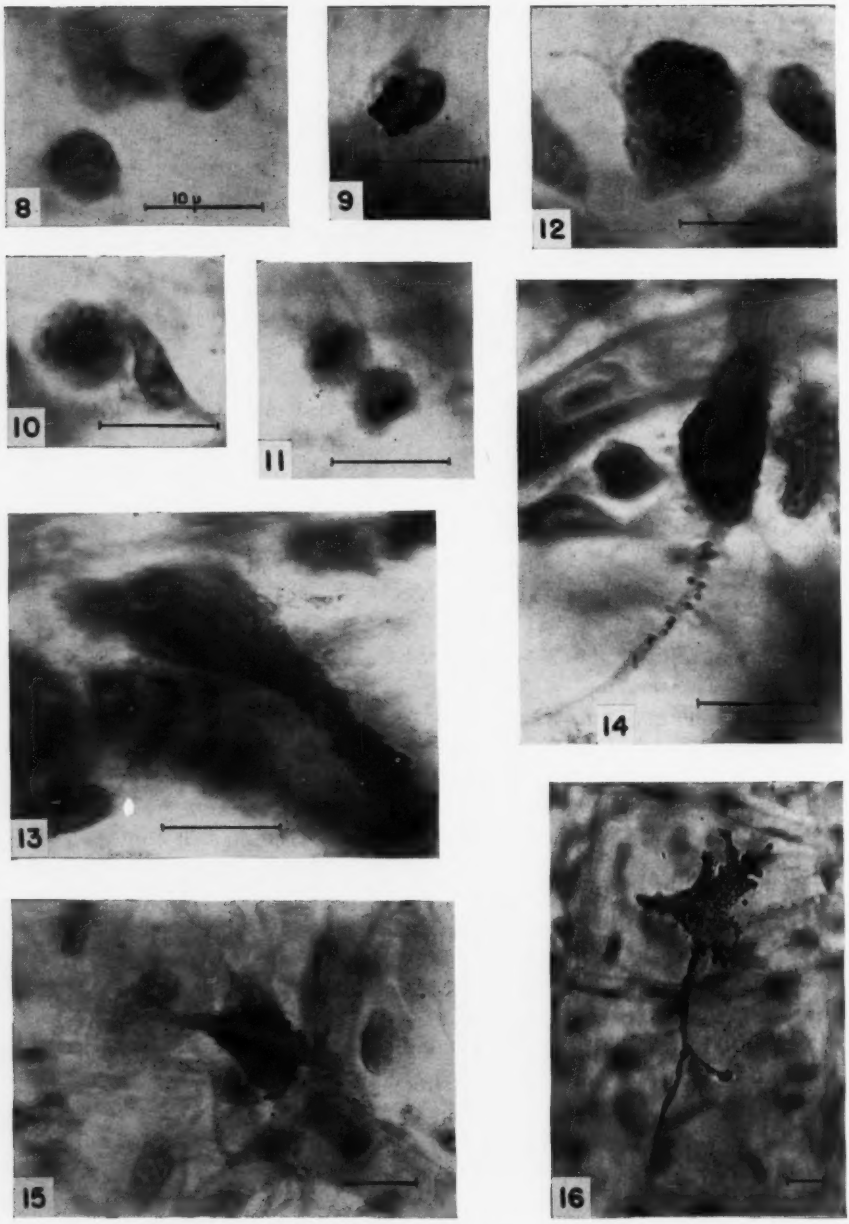
TABLE 3. SERIES XXVI AND XXVIII. RELATIVE CONCENTRATIONS OF MELANIN IN THE TAILS OF EXPERIMENTAL AND CONTROL FISH

Series and treatment	Prolactin, μg./gm.	Intermedin MSH units/gm.	Number of fish	Klett units[1]	
				Avg.	s
XXVI					
Prolactin	10	—	6	59.6	± 9.1
Intermedin	—	6000	6	51.5	± 8.5
Intermedin	—	600	4[2]	48.9	± 8.2
Prolactin+intermedin	10	600	5	66.2	±10.5
Saline controls	—	—	4[2]	48.6	± 2.8
XXVIII					
Prolactin+intermedin, in light	10	600	5	82.5	±17.5
Untreated controls, in light	—	—	4	59.5	±11.3
Prolactin+intermedin, in dark	10	600	5	87.8	± 6.4
Untreated controls, in dark	—	—	5	55.6	± 4.4

[1] Difference between mean readings for tissue extracts and solvent alone. The absolute values in Series XXVI are not comparable with those in Series XXVIII on account of the method of pretreatment (see text).

[2] One fish died 10 days before the end of the experiment, but the data have been included in the table since the assay value was in line with that of other members of the group and showed no stimulation.

phores were abundant in the latter and absent in the former. Whenever the state of dispersion of the pigment permitted accurate observation, the young melanophores were see to be binucleated (Fig. 16). Binucleated melanocytes were of frequent occurrence in the intermedin-treated fish (Figs. 13–15); no melanocytes with a single nucleus were observed. No mitoses were seen either in the melanophores or melanocytes. Both the skin of the control and that of the intermedin-treated fish contained numerous ovoid cells resembling macrophages, the cytoplasm was usually dense and the nucleus frequently lobed or twinned (Fig. 8). In the intermedin-treated fish such cells frequently contained melanin granules or balls of pigment (Fig. 9). One preparation from the control also contained a small group of melanin-containing macrophage-like cells, and it is possible that the granules had been ingested from a disintegrated melanophore although this could not be proven. The macrophage-like promelanocytes of the intermedin-treated fish were frequently observed in stages of mitosis, whether carrying melanin granules or not (Figs. 10–12). Sixteen such mitoses were counted in a total of 39 scale preparations, whereas only two were observed in 24 scales sampled from the control. Apparently intermedin stimulates the multiplication and differentiation of the propigment cells, and all stages in the transformation from ovoid, non-pigmented cells to melanocytes and young melanophores could be observed. The question of melanin ingestion versus melanin synthesis could not be clarified on the fixed material, but it would seem that both processes participate.



FIGS. 8–16. Stages in the multiplication and differentiation of melanophores in the ventral skin of hypophysectomized Fundulus, under the influence of intermedin (Series XXVI, 10μg./gm. dose). All preparations, except Figure 8, were taken from intermedin-treated fish; the scale (10 μ) indicates the magnification.

Series XXVIII

The question arose as to whether illumination affected the response to the hormones of melanogenesis. In order to answer this question an experiment was arranged with four groups of fish, two in the dark and two under standard conditions of illumination.

Protocol. Male fish taken in July, 1956, were hypophysectomized Aug. 22–23, and the experiment was started on Nov. 21, two months after operation. Fish scheduled for the "dark" tanks were kept therein for a period of two weeks before the beginning of injections. The illuminated fish were exposed to light from 20 watt fluorescent lamps (giving a somewhat brighter light than the 15 watt lamps employed in previous experiments) for 8 hours per day; the bottom of the tank was a dark slate grey. The "dark" tank fish were protected from light by a light-proof box, but it was necessary to expose them to a dim red light for one hour each day: 30 minutes in the morning for feeding, and 30 minutes in the afternoon for cleaning and, thrice weekly, injection. During the second and third weeks, on injection days, each anesthetized fish was exposed for a few seconds to a bright light in order to inspect the belly for the appearance of new melanophores which could not be seen under the red light. The control groups were untreated, since previous experiments had shown that saline injections merely impaired the viability of the fish without affecting the pigmentation. The experimental groups received a combination of prolactin (C. H. Li, a third preparation; 10 μg./gm. wt.) and intermedin (the same preparation used in Series XXVI; 1 μg./gm. wt.).

Results. The fish did not grow during the experiment, and the gonads were in total regression. Thyroids and anterior interrenals were not studied. Dorsal darkening and new melanophore formation appeared in both experimental groups (Tables 2 and 3). General darkening of the dorsal surface was evident at the end of the first week, in the illuminated group, and increased progressively. It is possible that there was a slight lag in the "dark" group, but this was doubtful, and the degree of dorsal darkening at the end of the experiment was the same in both groups. Careful inspection was made to determine the time of appearance of the new melanophores and the findings (Table 4) indicate a significant lag in the "dark" group. The mean time of appearance of new melanophores was 10 days in the light and 15 days in the dark, the difference is statistically significant ($P = <0.02, >0.01$).

Fig. 8. Macrophage-like propigment cells, with twin nuclei.
Fig. 9. The same, with a ball of ingested melanin.
Figs. 10 and 11. Unpigmented macrophages in stages of mitosis.
Fig. 12. A macrophage with melanin granules, in mitosis (anaphase).
Fig. 13. Early melanocyte with two nuclei and dispersed melanin granules.
Fig. 14. The same, with a ball of melanin pigment.
Fig. 15. Advanced melanocyte showing two nuclei and cell processes.
Fig. 16. Young melanophore, showing the two nuclei.

TABLE 4. SERIES XXVIII. DAY ON WHICH NEW MELANOPHORES WERE FIRST OBSERVED ON SIDES OF BELLY IN FISH TREATED WITH PROLACTIN + INTERMEDIN

Group	Serial No. of fish	Day after start of injections	Average
In light	12	11	
	17	11	
	21	11	
	22	9	
	32	9	10.2 ± 1.0
In dark	25	21	
	26	14	
	27	11	
	28	18	
	30	11	15.0 ± 1.6

The tails, removed at autopsy, were preserved in a mixture of 100% alcohol and formaldehyde (9:1), decalcified in 70% alcohol with 3% HNO_3 (3 days), dehydrated through 95% and 100% alcohol, and washed in two changes of ether during 16 hours to remove lipoid pigments. They were then dried and used two weeks later for the melanin assay, the results of which are given in Tables 2 and 3. There was no significant difference between the prolactin + intermedin-treated fish in the light and in the dark, although the mean value for the latter group was slightly greater. The control groups were alike. When the treated groups were compared with their corresponding controls, the increase in melanin was found to be significant; highly significant in the case of the "dark" group ($P = 0.001$), but only barely so in the case of the illuminated group ($P = $ ca. 0.05). The failure of the illuminated group to give decisive results, despite a mean difference of 23 Klett units as compared with the controls, can be attributed to the small number of fish and rather high individual variation. The results of the melanin assay support the visual findings and photographic records, which showed that the degree of darkening was essentially alike in fish kept in the dark and in the light.

It appears, from this experiment, that the influence of prolactin on melanin formation is essentially independent of illumination, but the proliferation of new melanophores in response to the action of intermedin is significantly augmented.

DISCUSSION

These experiments have demonstrated, beyond reasonable doubt, that highly purified prolactin, essentially free from active contaminations, stimulates an increased production of melanin in the melanophores of hypophysectomized Fundulus. No new pigment cells are formed. Highly purified intermedin, on the other hand, stimulates the proliferation of new melanophores which gradually spread over the normally pale ventral surface. The effective dose is high: ca. 100 MSH units per gram weight per injection, administered thrice weekly for a period of one month, barely suffices to elicit a minimal response. A dose of the order of 1000 MSH units, under

the same conditions, evokes a maximal response, since no greater effect was observed with 6000 MSH units. The threshold presumably resides in the inherent capacities of the melanophore producing system to respond. On the other hand, a dose of intermedin which is in itself insufficient can induce a maximal response when administered in conjunction with prolactin.

Two separate effects are apparently involved: melanin synthesis in already existing cells (prolactin) and new melanophore proliferation (intermedin). The former potentiates the latter. This hypothesis would be in agreement with the findings of Brantner (13) who showed that anterior lobe extracts, which presumably contained both intermedin and prolactin, were more effective than posterior lobe extracts in promoting a multiplication of melanophores in the bitterling, *Rhodeus amarus*. The prolactin preparation employed by Blair (25), which induced the formation of new melanophores in the toad, was apparently not contaminated with intermedin since no melanophore-dispersion was observed. However, the recipients were not hypophysectomized and a synergic action between exogenous prolactin and endogenous intermedin is not excluded. The experiments of Karkun and Mukerji (21) with ACTH-free intermedin appear to have shown that this hormone can induce the formation of new melanophores in frogs, but in this instance a synergism between exogenous intermedin and endogenous prolactin has not been excluded.

The concept of synergism between prolactin and other endocrine secretions receives support from the mammalian literature, although the relation of prolactin to melanogenesis has apparently not been investigated. Prolactin augments the action of androgens on the development of the prostate in the hypophysectomized-castrated rat, although it has no such effect when administered alone (36). Full lobulo-alveolar development of the mammary glands in hypophysectomized-castrated male rats requires the combined action of estrone, progesterone and growth hormone, in addition to prolactin (37).

There is some evidence, reviewed by Pickford & Atz (4), that a prolactin-like hormone may be present in the teleostean hypophysis. The discovery that this hormone plays some role in melanogenesis may give meaning to this otherwise inexplicable circumstance.

Anterior lobe hormones, other than prolactin, appear to have no effect on melanogenesis in Fundulus. Negative results were obtained with alpha corticotropin, thyrotropin, growth hormone and gonadotropins. Even highly purified corticotropin has been stated to possess a small inherent measure of melanophore-dispersing activity (38) but, if present, this was insufficient to stimulate the proliferation of these pigment cells. It should be noted that the preparation employed, alpha corticotropin, contained no prolactin. In Chavin's experiments on the goldfish (15), which gave results diametrically opposed to ours, it was not stated whether the ACTH preparation was free from prolactin, although it was known to contain inter-

medin. It is known that moderately well purified ACTH preparations, free from other anterior lobe hormones, may contain traces of prolactin (39), and although this hypothesis may seem far fetched it is one that deserves consideration. The failure of intermedin to induce melanogenesis in goldfish cannot be attributed to the dose, which was high, but the period of treatment (5 injections during 9 days) would have been barely sufficient for a response to be noted in Fundulus. If Chavin is correct in suggesting that ACTH is itself the factor which promotes melanization in goldfish, some other cause than possible contamination must be sought. The goldfish, unlike Fundulus, contained no melanin at the start of the experiment, but the significance of this is obscure. It should also be noted that Niu (40) described a transformation of lipophores into melanophores in the newt. The earliest melanin-containing cells observed by Chavin were irregularly dendritic melanocytes, but their anticedents were not investigated.

In Fundulus the newly formed melanocytes apparently differentiate from macrophage-like cells which frequently contain ingested melanin. These findings are in general agreement with the observations of Niu and Twitty (41) on the newt: at the time of metamorphosis the dermal melanophores degenerate and pigment granules are ingested by macrophages which, in turn, differentiate into melanocytes and give rise to the new epidermal melanophores. Twitty (42) has answered some of the criticisms of this interpretation, which nevertheless remains highly controversial. The hypothesis that the ingestion of products derived from disintegrating melanophores promotes melanin synthesis, and stimulates the process of transformation, may have some bearing on the results in Fundulus. It was repeatedly observed that new melanophores appeared first along the sides of the belly, adjacent to areas that were already pigmented, and it seems possible that macrophage transformation may have been promoted by a contribution of melanin from these regions. The fact that intermedin, in the absence of prolactin, causes little or no increase in the total amount of melanin suggests that the new melanophores may be acquiring their pigment in large part from the disintegration of old ones. It is possible, however, that continued loss of melanin was approximately balanced by new melanin synthesis.

Treatment with intermedin increased the rate of mitosis in the macrophage-like propigment cells, but no mitoses were observed in the melanocytes or differentiated melanophores. Niu and Twitty (41) observed frequent mitoses in the melanophores of the newt, as well as in the precursor cell types. The lack of mitosis in the melanocytes and melanophores of Fundulus may be related to the binucleate condition of these cells which is established at an early stage and, presumably, might preclude further cell multiplication.

Comparison of the results of experiments on illuminated fish with those kept in darkness introduces yet another factor into the problem of melano-

genesis in Fundulus. Although illumination against a dark background favors melanophore dispersion, there was no difference in the amount of melanin synthesis, under the influence of prolactin+intermedin, in the illuminated and non-illuminated groups. On the other hand, illumination significantly promoted the rate of proliferation of new melanophores, and a primary effect of light upon the skin is indicated. However, a possible role of melanophore-dispersing innervation is not excluded. Parker (2) suggested that conditions which favor melanophore dispersion might stimulate dormant melanophores through trophic action resulting from the continuous release of a melanophore-dispersing neurohumor from the nerve terminals. This action would be at a minimum in fish that were kept in darkness. It should also be noted that the illuminated fish were in a constant state of activity during the artificial day, whereas the fish in the dark tanks remained relatively quiescent. It seems possible that increased metabolism might play some role in accelerating the speed of the response.

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SUMMARY

Experiments were made on hypophysectomized *Fundulus heteroclitus* injected thrice weekly for one month at 20° C. Alpha corticotropin had no effect on pigmentation at doses of 1 μg. or 10 μg./gm. wt./injection. Highly purified prolactin (35 I.U./mg.), free from intermedin and ACTH, stimulated melanin synthesis in already developed melanophores at doses of 1 μg. or 10 μg./gm. wt./injection, but no new melanophores were formed. Highly purified intermedin, ACTH-free, caused little or no increase in melanin synthesis but promoted the proliferation of new melanophores which spread over the belly. The threshold dose for this response was ca. 100 MSH units/gm. wt./injection; a maximal response was elicited by ca. 1000 MSH units. The response to a low dose of intermedin was potentiated by simultaneous administration of prolactin. Illumination promoted the proliferation of melanophores, in response to combined treatment with intermedin+prolactin, but had no significant effect on the increased production of melanin. New melanophores differentiated from macrophage-like propigment cells already present in the dermis.

REFERENCES

1. Odiorne, J. M.: Special Publ. N.Y. Acad. Sci. "The Biology of Melanomas" **4**: 288. 1948.

2. Parker, G. H.: Animal Colour Changes and Their Neurohumors, Cambridge Univ. Press. 1948.
3. Abramowitz, A. A.: *J. Pharm. exp. Therap.* **69**: 156. 1940.
4. Pickford, G. E. and J. W. Atz: The Physiology of the Pituitary Gland of Fishes, N.Y. Zool. Soc. 1957.
5. Healey, E. G.: *Z. vergl. Physiol.* **27**: 545. 1940.
6. Healey, E. G.: *Bull. Animal Behav.* **6**: 5. 1948.
7. Matthews, S. A.: *Biol. Bull.* **64**: 315. 1933.
8. Osborn, C. M.: *Biol. Bull.* **81**: 341. 1941a.
9. Osborn, C. M.: *Biol. Bull.* **81**: 352. 1941b.
10. Veil, C.: *C. R. Acad. Sci.*, Paris **201**: 914. 1935.
11. Veil, C.: *J. Physiol. Path. gén.* **34**: 824. 1936.
12. Abramowitz, A. A.: *Biol. Bull.* **73**: 134. 1937.
13. Brantner, G.: *Z. vergl. Physiol.* **33**: 324. 1956.
14. Ito, M.: *Tohoku J. exp. Med.* **55**: Suppl. 1: 1. 1952.
15. Chavin, W.: *J. exp. Zool.* **133**: 1. 1956a.
16. Hu, F. and W. Chavin: *Anat. Rec.* **125**: 600. 1956.
17. Müller, J.: *Z. vergl. Physiol.* **35**: 1. 1940.
18. Pickford, G. E.: *Endocrinology* **55**: 589. 1954b.
19. Pickford, G. E.: *Bull. Bingham oceanogr. Lab.* **14**(2): 6. 1953b.
20. Pickford, G. E.: *Endocrinology* **55**: 274. 1954a.
21. Karkun, J. N. and B. Mukerji: *Indian J. med. Res.* **41**: 467. 1953.
22. Chavin, W.: *Anat. Rec.* **125**: 603. 1956b.
23. Dalton, H. C. and Z. P. Krasner: *Anat. Rec.* **120**: 720. 1954.
24. Blacher, L. J.: *Trans. Lab. exp. Biol. Zoopark Moscow* **3**: 79. 1927.
25. Blair, A. P.: *J. exp. Zool.* **103**: 365. 1946.
26. Lerner, A. B., K. Shizume and I. Bunding: *J. Clin. Endocr. Metab.* **14**: 1463. 1954.
27. Lerner, A. B.: *Amer. J. Med.* **19**: 902. 1955.
28. Lerner, A. B. and Y. Takahashi: *Recent Progr. Horm. Res.* **12**: 303. 1956.
29. Pickford, G. E.: *Bull. Bingham oceanogr. Lab.* **14**(2): 5. 1953a.
30. Burden, C. E.: *Biol. Bull.* **110**: 8. 1956.
31. Foster, M., R. Cook and T. A. Stamas: *J. exp. Zool.* **132**: 1. 1956.
32. Shizume, K., A. B. Lerner and T. B. Fitzpatrick: *Endocrinology* **54**: 553. 1954.
33. Grant, W. C. and J. A. Grant: *Anat. Rec.* **125**: 604. 1956.
34. Li, C. H., F. I. Geschwind, A. L. Levy, J. I. Harris, J. S. Dixon, N. G. Pon and J. O. Porath: *Nature* **173**: 251. 1954.
35. Cole, R. D. and C. H. Li: *J. Biol. Chem.* **213**: 197. 1955.
36. Grayhack, J. T., P. L. Bunce, J. K. Kearns and W. W. Scott: *Bull. Johns Hopkins Hosp.* **96**: 154. 1955.
37. Lyons, W. R., R. E. Johnson, R. D. Cole and C. H. Li: *Internat. Symp.* "The Hypophyseal Growth Hormone," Blakiston: 416. 1955.
38. Dixon, H. B. F.: *Biochim. biophys. Acta* **19**: 392. 1956.
39. Payne, R. W., M. S. Raben and E. B. Astwood: *J. Biol. Chem.* **187**: 719. 1950.
40. Niu, M. C.: *J. exp. Zool.* **125**: 199. 1954.
41. Niu, M. C. and V. C. Twitty: *J. exp. Zool.* **113**: 633. 1950.
42. Twitty, V. C.: Pigment Cell Growth, Academic Press: 26. 1953.

SELECTIVE INHIBITION BY CORTISONE OF ADRENAL CORTICAL INCORPORATION OF C^{14} FROM LABELLED BACTERIAL POLYSACCHARIDE[1,2]

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AFTER the injection of C^{14} labelled polysaccharide complexes from *Klebsiella pneumoniae* in the guinea pig, the adrenal cortex has the highest tissue concentration of isotope (1). In contrast, the adrenals of the mouse, rat and rabbit incorporate minor amounts of C^{14} (2). With the exception of the adrenal, the tissue distribution of C^{14} in the four animal species is similar to that observed with dye-tagged protein antigens (3) and with *K. pneumoniae* capsular polysaccharide identified in mouse tissue with fluorescent antibody (4). The C^{14} derived from the bacterial polysaccharide persists in the adrenal for at least two months (1). The exact nature of the chemical incorporation in the adrenal has not been determined; only a minor amount of the C^{14} within the adrenal is in polysaccharide or lipid which can be extracted from the adrenal (5), and only 17% of the extracted adrenal polysaccharide possesses the haptenic properties of the injected substance, in comparison with 45% in polysaccharide fraction from the spleen and 45 to 62% in fractions from the liver. In addition, the rate of C^{14} uptake in the adrenal differs from that in other organs; the C^{14} incorporation by the adrenal continues to increase over a period of several days while maximum concentration in other organs occurs within hours (1, 5).

The present report concerns the modification of the adrenal uptake of C^{14} from labelled bacterial polysaccharide by the injection of ACTH and various steroids involved in the biosynthetic pathway of adrenal corticosteroids.

METHODS AND MATERIALS

Young guinea pigs of either sex and weighing 200–250 gm. were given water, Purina rabbit pellets and daily supplements of cabbage. A polysaccharide complex extracted with .01N NaOH from *K. pneumoniae*, type B (1, 5, 6), was labelled by biosynthesis

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with 1-C^{14} acetate (1, 5). This extract was haptenic but not antigenic and contained 4.3% nitrogen, 6.8% protein and 35% reducing sugar, as glucose, after hydrolysis (5).

All animals, including controls, were given a single intravenous injection of the labelled, alkaline-extracted polysaccharide from *K. pneumoniae* and sacrificed seven days later. The 1% solution of the polysaccharide was prepared in 0.85% NaCl, autoclaved and 0.2 ml. (2.0 mg.) injected into a transilluminated ear vein.

The following substances were injected subcutaneously each day in the indicated quantities: ACTH, 5 and 10 I.U. in 1% gelatin; pregnenolone (Δ^5 pregnen-3-ol-2 0-one), 10 mg. in sesame oil; progesterone, 10 mg. in sesame oil; 17α hydroxy-11-desoxycorticosterone (Reichstein's substance S), 10 mg. in sesame oil; cortisone, 2.5 and 10 mg. in Merck vehicle #1; cortisone, 10 mg. plus ACTH, 10 I.U.; and corticosterone (Kendall's compound B), 3.0 mg. per 100 gm. body weight in Merck vehicle #1. Vehicle #1 was also given to a group of guinea pigs.

Daily injections of the above steroids and ACTH were begun seven days before and continued for seven days after the single intravenous injection of labelled polysaccharide. Guinea pigs in three additional groups received 2.5 mg. cortisone per day and 10 I.U. ACTH per day beginning the day of the polysaccharide injection and continuing until the animals were sacrificed one week later. A control group received ACTH plus cortisone but no polysaccharide. After the injection of the labelled polysaccharide, urine and respired CO_2 were collected at various intervals and the C^{14} content determined as previously described (5).

Upon sacrificing the animals, blood was removed by cardiac needle puncture and immediately separated by centrifugation into plasma and cells. Total plasma volume was considered as 5% of the body weight at autopsy. The liver, spleen and adrenals were weighed and the per cent dry weight determined by comparison of wet weight of a sample with the weight after drying overnight in a 100° C oven. Small portions of various organs and tissues (5) were ground with distilled water in a Potter homogenizer, plated and counted with a windowless flow counter. C^{14} content of tissues is expressed as the per cent of injected dose a) per gram of dry tissue and, b) per organ for the liver, spleen and adrenals. The wet weights of the three organs are given as the per cent of body weight at autopsy (Table 1).

RESULTS

Cortisone markedly reduces the adrenal uptake of C^{14} from the labelled bacterial polysaccharide (Fig. 1). This occurs only when the cortisone is injected prior to the polysaccharide. The effect of 2.5 mg. was almost as great as 10 mg. of cortisone per day. Except for a slight reduction of C^{14} incorporation by the adrenal after prior injections of 17α hydroxy-11-desoxycorticosterone, the other steroids and ACTH were without effect. The vehicle #1 alone did not alter the uptake of C^{14} by the adrenal.

The considerable reduction in adrenal C^{14} uptake with 2.5 mg. of cortisone per day occurred without accompanying reduction in relative adrenal weight (Table 1). Daily injections of ACTH begun either one week before or the same day as the single intravenous bacterial polysaccharide, resulted in a relative increase in adrenal weight. The per cent of the injected C^{14} incorporated in these adrenals was slightly decreased per gram of tissue, but, due to the increased weight of these organs, the total incorporation of C^{14} was augmented. When ACTH was given with cortisone, the cortisone effect upon the adrenal was not observed: ACTH plus cortisone resulted in

the same adrenal C^{14} content and the same relative weights as in the control groups.

Pretreatment with ACTH and cortisone, given individually or in combination, increased the relative liver weight and decreased the C^{14} uptake per gram of dry liver (Table 1). The gain in relative liver weight varied with the dosage of the hormones but all of the employed quantities of cortisone and ACTH gave similar reductions in the C^{14} per gram of tissue. The greatest increase in liver/body weight occurred after injection of 10 mg. cortisone and 10 mg. cortisone plus 10 I.U. ACTH per day. In considering the quantitative aspects of C^{14} distribution in tissue, the total in-

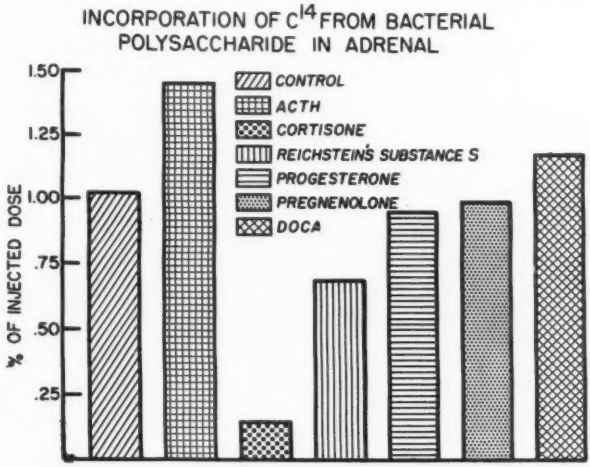

FIG. 1

corporation per organ is of significance. The C^{14} incorporation per organ is dependent upon the actual rather than the relative weight. In animals given the larger doses of cortisone alone or with ACTH, the livers were so increased in actual weight that the reduction in C^{14} per unit weight was overcome, thereby yielding a modest increase in total C^{14} per organ.

The per cent dry weight of the liver, spleen, and adrenal showed little change after the injection of various steroids and ACTH. However, there is some evidence that alterations in body fluids may occur. Animals receiving both cortisone and ACTH developed considerable ascites by the time of sacrifice two weeks after the initiation of the daily injections. The ascitic fluid contained little C^{14}, reflecting the low plasma levels in all groups at this time. Animals receiving cortisone plus ACTH, but no polysaccharide, developed smaller but definite quantities of protein-rich ascitic fluid. Some of the animals given cortisone plus ACTH showed numerous small areas of fat necrosis in the abdominal but not in the subcutaneous fat. The areas of fat necrosis occurred more frequently in the larger or more mature ani-

TABLE 1. THE EFFECT OF STEROIDS AND ACTH UPON THE ADRENAL UPTAKE OF C^{14} FROM A SINGLE INTRAVENOUS INJECTION OF LABELLED BACTERIAL POLYSACCHARIDE

	Number of guinea pigs	Daily hormone injections prior to bacterial polysaccharide	Amount hormone per day	Adrenals			Spleen			Liver			Cervical lymph node %/gm.	Mesenteric lymph node %/gm.
				% *organ* body wt. (wet)	%/gm.	% of total dose	% *organ* body wt.	%/gm.	% of total dose	% *organ* body wt.	%/gm.	% of total dose		
Controls	8	—	—	.043 ± .009	25.60 ± 4.73	1.02 ± 0.26	.144 ± .008	5.11 ± 1.55	0.47 ± .14	4.19 ± .67	3.16 ± 1.12	10.68 ± 3.45	2.20 ± .95	0.61 ± .44
Cortisone	4	0	2.5 mg.	.060 ± .017	22.88 ± 1.73	1.01 ± .20	.145 ± .010	6.63 ± .54	0.48 ± .02	5.10 ± 1.16	2.24 ± .39	6.71 ± 1.86	0.55 ± .21	0.51 ± .37
Cortisone	7	7	2.5 mg.	.048 ± .008	5.88 ± 1.54	0.27 ± .10	.221 ± .059	2.83 ± .96	0.40 ± .12	4.66 ± .74	1.96 ± .39	7.39 ± 1.47	0.84 ± .73	0
Cortisone	5	7	10 mg.	.039 ± .003	3.71 ± .42	0.14 ± .04	.170 ± .045	6.44 ± 2.71	0.59 ± .16	7.66 ± .53	1.78 ± .28	10.51 ± 1.48	1.21 ± .94	0.35 ± .10
ACTH	5	7	5 I.U.	.085 ± .017	17.07 ± 2.70	1.29 ± .44	.161 ± .026	3.15 ± .42	0.32 ± .02	4.83 ± .46	1.86 ± .36	7.84 ± 1.08	0.62 ± .33	0.14
ACTH	5	7	10 I.U.	.084 ± .030	17.16 ± 3.69	1.46 ± .48	.153 ± .021	3.07 ± 1.68	0.30 ± .10	5.11 ± 1.04	1.72 ± .75	6.92 ± 1.86	0.84 ± .74	0.27
ACTH	8	0	10 I.U.	.064 ± .011	20.57 ± 3.42	1.42 ± .46	.154 ± .017	5.30 ± .98	0.55 ± .14	4.09 ± .45	3.58 ± 1.33	12.79 ± 4.25	1.84 ± .94	0.91 ± .48
ACTH and Cortisone	5	7	10 I.U. 10 mg.	.054 ± .009	16.40 ± 1.41	1.43 ± .17	.123 ± .023	8.01 ± 1.41	0.98 ± .27	6.19 ± .38	1.98 ± .13	14.98 ± 1.52	0.51 ± .48	0.31 ± .16
DCA	4	7	10 mg.	.043 ± .008	19.59 ± 5.44	1.20 ± .36	.275 ± .039	3.46 ± 1.07	0.78 ± .19	4.07 ± .46	4.46 ± 1.38	18.50 ± 2.22	1.65 ± .17	1.42 ± .89
Pregnenolone	5	7	10 mg.	.045 ± .014	22.48 ± 5.83	0.99 ± .20	.349 ± .109	3.15 ± .94	0.73 ± .23	4.84 ± .75	3.95 ± .99	17.80 ± 4.48	3.78 ± .97	0.89 ± .37
Progesterone	5	7	10 mg.	.047 ± .011	21.02 ± 3.58	0.93 ± .26	.290 ± .132	3.87 ± 1.58	0.68 ± .12	4.94 ± .25	3.25 ± .85	14.60 ± 3.56	2.81 ± 1.02	0.90 ± .42
Substance S	5	7	10 mg.	.042 ± .013	18.04 ± 4.36	0.72 ± .20	.208 ± .032	4.65 ± 1.35	0.72 ± .22	4.83 ± .44	3.76 ± 1.03	16.40 ± .98	2.96 ± 1.14	1.01 ± .57
Corticosterone	5	7	10 mg.	.051 ± .014	21.60 ± 4.70	1.08 ± .32	.222 ± .012	3.37 ± 1.04	0.50 ± .18	4.33 ± .40	3.42 ± .85	9.96 ± .98	2.75 ± .68	1.55 ± .83

mals, but did not appear associated with the quantity of ascitic fluid or dependent upon the injection of polysaccharide.

There was less C^{14} in the lymph nodes of animals given cortisone. It is of interest that little histologic change was observed in the lymph nodes or thymuses of guinea pigs given the various steroids or ACTH.

DISCUSSION

The present study has shown that the high adrenal uptake of C^{14} from a labelled bacterial polysaccharide is markedly depressed by pretreatment with cortisone; this effect of cortisone does not occur when ACTH is given concomitantly. In the subsequent paragraph the above observations are considered in relation to some of the biologic interactions of the bacterial polysaccharide, the cortisone and the ACTH.

The bacterial polysaccharide used in this study is a non-lethal, non-antigenic, hapten. Unlike some related bacterial extracts, this polysaccharide, when given intravenously, does not produce bile precipitate, ascites, or basophilia of the hepatic cord cells (5). However, it does produce acute arthritic lesions (5). After a single intravenous injection of the polysaccharide the adrenal is engorged during the first day, the plasma 17 hydroxycorticosteroids are markedly elevated at 4 to 6 hours and the adrenal ascorbic acid is reduced (7). It is not known if the adrenal steroidogenesis is altered during the long residence of the C^{14} in the adrenal.

Since the nature of the chemical incorporation of the C^{14} in the adrenal is unknown, the mechanism by which cortisone depresses the C^{14} uptake by the adrenal is as obscure as the other biologic effects of cortisone such as cold-stress survival, altered muscular work, liver glycogen deposition, "anti-inflammatory" activity, thymolysis, eosinopenia, etc.

The pituitary-adrenal feed-back system of Sayers (8) may explain the suppression of adrenal C^{14} uptake by cortisone. According to this concept, exogenous cortisone and other "anti-inflammatory" steroids reduce the output of ACTH by the pituitary which then leads to diminished 17 hydroxysteroidogenesis by the adrenal. When exogenous ACTH is supplied, the adrenal steroidogenesis is maintained, thus explaining the findings in the present experiment. Adrenal cortical function would, thereby, appear to be associated with the incorporation of C^{14}.

Some evidence suggests that exogenous ACTH and cortisone may alter more than 17 hydroxycorticosteroid formation by the adrenal. ACTH and cortisone in combination produce mild ascites but, when given separately, do not. Further studies are required to elucidate such possible explanations for this effect as excessively high plasma 17 hydroxycorticoids, increased "mineralo-corticoids" or aldosterone, altered utilization or metabolism of such steroids, and changes in capillary permeability.

Since urinary loss of the isotope is similar for control and treated groups,

cortisone would not appear to lower the adrenal C^{14} by enhancing urinary excretion. In addition, the C^{14} incorporation by tissues other than the adrenal are affected far less than the adrenal.

The C^{14} incorporated by the adrenal reaches its maximum after several days in contrast to the more rapid uptake in other organs (1, 5). Thus, changes in the distribution, metabolism, depolymerization of the polysaccharide in other organs could readily influence the subsequent uptake of C^{14} containing moieties by the adrenal cortex. Since much of the polysaccharide is found in the organs of the "reticuloendothelial system" one might speculate that the phagocytes are altered by cortisone. Evidence against this is the finding that ACTH prevents rather than enhances the effect of exogenous cortisone. This argument could be refuted if ACTH were found to have actions which oppose those of cortisone upon reticuloendothelial or other cells. Some of the decrease in C^{14} per gram of liver with ACTH and cortisone may be due to the trophic and glycogen accumulating actions; the increased liver weight may counter balance the decrease per gram (Table 1) and yield an increase in total C^{14} in the liver. While both ACTH and cortisone have similar actions upon the C^{14} content of the liver, only cortisone markedly depresses the adrenal C^{14}. Local action of cortisone upon the reticuloendothelium of the adrenal would not seem to be of significance; the reticuloendothelium of the adrenal is less abundant and apparently less active than other phagocytic cells of the reticuloendothelial system (9). The apparent transmittal of an occasional particle from the sinusoidal phagocytes to the cortical cells (9) could scarcely explain the marked concentration of C^{14} in the adrenal.

Other investigators have noted an apparent relation between adrenal steroidogenesis and susceptibility to adrenal injury. Frenkel (10) has observed that the adrenal necrosis in hamsters infected with *Besnoitia jellisoni* may be related to the amount of locally available glucocorticoids. The infected hamsters show less adrenal necrosis when treated with cortisone, hydrocortisone, corticosterone and 11-dehydrocorticosterone (compound A). The *Besnoitia* organism also proliferate about the injection site of exogenous cortisone, cortisol and 11-dehydrocorticosterone but not about corticosterone (17-hydroxy-11-desoxycorticosterone), DCA, testosterone proprionate, or epinephrine in oil. However, exogenous porcine ACTH did not support the proliferation of *Besnoitia* in the adrenals of hypophysectomized animals.

Another example of the apparent interrelation of adrenal steroidogenesis and adrenal injury has been described by Tonutti (11). Diphtheria toxin produces hemorrhagic "necrosis" in the adrenal of the guinea pig only when the pituitary is present or when ACTH is supplied to hypophysectomized animals. Other toxins may produce the adrenal injury in hypophysectomized animals.

SUMMARY

In the guinea pig, the highest level of C^{14} derived from a labelled bacterial polysaccharide is in the adrenal cortex. The chemical nature of the C^{14} uptake by the adrenal is obscure but may be a significant index of "anti-inflammatory" or "glucocorticoid" activity of steroids. The adrenal incorporation of C^{14} is markedly depressed by cortisone, slightly depressed by 17-hydroxy-11-desoxycorticosterone but unaltered by corticosterone, 11-desoxycorticosterone, progesterone, or pregnenolone. The action of cortisone on the adrenal occurs in pre-treated animals and is prevented by concomitant administration of ACTH. Some possible interactions of the bacterial polysaccharide, cortisone and ACTH are discussed.

Acknowledgments

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REFERENCES

1. Jones, R. S. and Y. Carter: *Proc. Soc. Exper. Biol. & Med.* **90:** 148. 1955.
2. Jones, R. S. and Y. Carter: *Fed. Proc.* **15:** 520. 1956.
3. Latta, H., D. Gitlin and C. A. Janeway: *Arch. Path.* **51:** 260. 1951.
4. Hill, A. G. S., H. W. Deane and A. H. Coons: *J. Exper. Med.* **92:** 35. 1950.
5. Jones, R. S. and Y. Carter: *Arch. Path.* **63:** 472. 1957.
6. Wong, S. C.: *Proc. Soc. Exper. Biol. & Med.* **38:** 107. 1938.
7. Eik-Nes, K., Y. C. Mayne and R. S. Jones: Unpublished observations.
8. Sayers, G.: *Physiol. Rev.* **30:** 241. 1950.
9. Cappell, D. F.: *J. Path. & Bact.* **32:** 595. 1929.
10. Frenkel, J. K.: *J. Exper. Med.* **103:** 375. 1956.
11. Tonutti, E.: In *Mechanisms of Inflammation* (G. Jasmin and A. Roberts, editors). Montreal, Acta, Inc., Medical Publications, 1953, 111.

NOTES AND COMMENTS

SIMPLIFIED PREGNANEDIOL METHOD: FORMALDEHYDE INHIBITION OF URINE CHROMOGEN FORMATION DURING ACID HYDROLYSIS

Antunes (1) reported that the addition of formalin to urine prior to acid hydrolysis prevented the formation of interfering chromogens. The subsequent ether extracts were so colorless that he could omit the extract blank in the determination of 17-ketosteroids. It was decided to add formalin to urine before hydrolyzing the pregnanediol complex. If a colorless toluene extract could be obtained it would eliminate the need for decolorization by charcoal or by NaOH pellets.

The quantity of formaldehyde was varied from 0.5 cc. to 2.0 cc. per 250 cc. of urine. The larger quantities frequently caused the formation of a white fluffy precipitate which

TABLE 1. RECOVERY OF PREGNANEDIOL FROM URINE WITH AND WITHOUT ADDED FORMALDEHYDE. (0.5 CC. PER 250 CC. OF URINE)

Urine specimen	Control	Formaldehyde	Formalin/Control—%
1	5.0 mg.	5.0 mg.	100
2	4.7 mg.	4.2 mg.	89
3	19.2 mg.	23.0 mg.	120
4	19.8 mg.	18.0 mg.	90
5	120.0 mg.	96.0 mg.	
	125.0 mg.	98.0 mg.	
	115.0 mg.	101.0 mg.	82
6	68.0 mg.	50.0 mg.	
	62.0 mg.	56.0 mg.	
	62.0 mg.	49.0 mg.	81
7	67.0 mg.	67.0 mg.	
	66.0 mg.	66.0 mg.	100
8	42.0 mg.	40.0 mg.	
	45.0 mg.	41.0 mg.	
	41.0 mg.	41.0 mg.	95

accumulated at the toluene-water interface and made a clean separation difficult. Smaller quantities of formaldehyde were equally effective in preventing color formation, so 0.5 cc. of formaldehyde per 250 cc. of urine was adopted as a standard quantity.

To some urine aliquots, formaldehyde was added, then 15 volumes per cent of hydrochloric acid. (The formaldehyde must be added to the urine before the HCl is added.) This was overlaid with 60 cc. of toluene and boiled for 15 minutes under reflux. The formaldehyde was omitted from the other aliquots but they were acidified and boiled in a similar manner. All the toluene extracts were washed twice with normal NaOH and twice with water. The toluene was then evaporated to dryness. The residues were taken up in 10 cc. of 95% ethanol. The formaldehyde treated urine extracts were colorless and were

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immediately precipitated by the addition of four successive volumes of hot 0.1 NaOH. The other ethanol solutions were highly colored and charcoal was added. These solutions were then filtered to remove the charcoal and the filters were washed quantitatively with ethanol. The alcohol filtrates and washings were then evaporated to a 10 cc. volume and the pregnanediol was precipitated. The entire procedure is basically that of Guterman and Schroeder (2) except for substitution of charcoal for the methanolic NaOH which they used for decolorization.

In table 1, the recovery of pregnanediol from urine aliquots in which the formation of chromogens was prevented by the addition of formaldehyde is compared to the recovery from those aliquots which were decolorized by the use of charcoal.

Whether the actual pregnanediol recovery is reduced or whether other sulphuric acid chromogens are preferentially eliminated by the addition of formaldehyde is not known since the pregnanediol complex does not lend itself to any specific quantitative chemical estimation. Our experience in the past six months has given us confidence that any possible small loss in pregnanediol recovery is offset by the advantage gained in shortening the procedure.

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REFERENCES

1. Antunes, L. N.: *J. Clin. Endocrinol. & Metab.* **16:** 1125–1126. 1956.
2. Guterman, H. S. and M. S. Schroeder: *J. Lab. & Clin. Med.* **33:** 356–366. 1948.

PRESIDENTIAL ADDRESS

LAWSON WILKINS

May 31, 1957

FOREWORD

The following address is not to be considered as a history of endocrinology dedicated to giving detailed credit and priorities to the various leaders and workers in the field. It attempts merely to paint the picture of the changing concepts and methods of endocrinology and to show the close interlocking of the many disciplines. Due to lack of time the names of many leading contributors have not been mentioned. For these many omissions the writer humbly apologizes and hopes that no personal offense is taken.—L. W.

Members and Guests of The Endocrine Society:

I KNOW from my own experience that at this time of the evening you, one and all, begin to think of Bed or of Bar while you consider it your painful duty to listen politely, but somewhat drowsily, while a President ruminates about Past, Present or Future. I shall risk being accused of senility by turning my thoughts to the Past of Endocrinology.

I cannot resist the temptation to look back to the years when I was a Medical Student and House Officer and to recall the status of endocrinology at that time. I was a little surprised to recall that the founding in 1918 of The Endocrine Society, then known by the tongue-twisting title of The Association for the Study of Internal Secretions, coincided with the year of my graduation and that the first President was my Professor of Medicine, Dr. Lewellys F. Barker. During my student and hospital days there was a great deal of talk and enthusiasm about the endocrine glands and their relation to certain diseases was well recognized. Indeed, there was a feeling of tense excitement and there were interesting speculations concerning marvelous discoveries which probably lay ahead in the mystic future.

ENDOCRINOLOGY IN 1920

In 1920 the various *thyroid disorders* were recognized and diagnosed clinically almost as well as they are today. Thyroid substance had been used therapeutically ever since 1893 when Osler administered it to the first cretin in this country one year after MacKenzie and Fox, in England, had demonstrated its effectiveness. The BMR was being used in diagnosis. Thyroidectomies were being performed but there was still a high mortality since Plummer did not demonstrate the preoperative value of Lugol's solution until 1923. Marine was carrying out, in Akron, Ohio, large scale demonstrations of the value of iodine in the prevention of endemic goiter. Kendall had just announced the isolation of crystalline thyroxine.

The relation of the islets of Langerhans to diabetes mellitus had been demonstrated by von Mering and Minkowski in 1889. In histology, we

students were shown the difference between alpha and beta cells, but we did not know their respective functions. The importance of diet and the role of fat in ketosis and acidosis were understood. We had begun to measure CO_2-combining power and we gave our acidotic patients Na-bicarbonate. We had no specific therapy and had never heard of the importance of K.

We knew that the tiny *parathyroid glands* were important in regulating serum calcium because MacCallum and Voegtlin at Hopkins in 1908 had produced hypocalcemic tetany by parathyroidectomizing dogs. In many other places this relationship was denied and there was much talk about the convulsive effects of guanidine and the detoxifying action of the parathyroids. But we were loyal to our Alma Mater and all for MacCallum and calcium. We were, however, confused a bit by some of the other types of hypocalcemic tetany, and vitamin D had not yet been discovered.

Sex and the *sex glands* naturally had been of interest since ancient times and the effects of both sexual precocity and castration on somatic growth as well as sexual development had been well described. Although Leydig had observed the interstitial cells of the testes in 1850, their function was still unknown. Prenant in 1898 had suspected the *corpus luteum* of being an endocrine organ and subsequently the fact that its presence was required for the implantation of the ovum and the maintenance of pregnancy had been established. Gynecologists had studied extensively the endometrial changes of the menstrual cycle and Papanicalou had begun to study the cyclic alterations of the vaginal epithelium.

The *adrenals* were looked upon as glands of considerable importance; and the embryological and functional differences between cortex and medulla were understood. The first pure hormone, epinephrine, had been isolated in 1899 by our professor of pharmacology, Abel; and it had been synthesized in 1905. Its pharmacologic actions had helped greatly in unravelling the mysteries of the sympathetic and parasympathetic systems. Ampules of adrenalin were at hand on every ward for us internes to use in the treatment of all sorts of shock and we often injected it heroically into the vertricles when the heart stopped beating. We were taught the great importance of the adrenal medulla in preparing the animal to meet acute stress and in adapting to special emergencies as pointed out by Walter Cannon. "Diseases of Adaptation" were not part of our curriculum.

The diverse manifestations of adrenal disorders puzzled us very much. On the one hand, our professors showed us not infrequently patients with the disease Addison had described with its pigmentation, asthenia, wasting, hypotension, and inevitable death; but offered no satisfactory explanation for these symptoms. On the other hand, it had been known since the paper of Bulloch and Sequeira in 1905 that pseudohermaphroditism, virilization and hirsutism were sometimes associated with tumor or enlargement of the adrenal cortex. Cushing's syndrome had not been heard of.

The *pituitary gland,* which Vesalius had accused of secreting nasal mucus, in 1920 attracted attention principally because of its alleged effects on growth. Pierre Marie in 1886 had considered acromegaly due to destruction of the pituitary by a tumor. However, in 1916 Erdheim had emphasized the association of dwarfism and sexual infantilism with destructive lesions of the pituitary. We were quite willing to accept his hypothesis that the pituitary accelerated growth, because our own teachers Samuel Crowe and Harvey Cushing in 1910 had produced dwarfed dogs by hypophysectomy. Skeptics elsewhere contended that the dwarfing probably was due to general debility and malnutrition resulting from the operation and nearly 20 years were to pass before the growth hormone theory was proved by replacement therapy in rats by P. E. Smith and in dogs by Herbert Evans and his colleagues.

The anterior pituitary had not yet assumed its role as the omnipotent "master gland" or the magnificent homeostatic regulator of the endocrine system, although it was strongly suspected that it might have other effects than those on growth and some close relationship to the nervous system. It was recognized that there were interrelationships between the various endocrine glands. However, since potent hormones had not been isolated, and implantation of bits of glandular tissue often gave confused and conflicting results, the interrelationships were based largely on extirpation experiments, or clinical pathologic observations, in which it was found that when a certain gland was removed, some of the other glands hypertrophied while others atrophied. In 1910, Biedl had collected and described these numerous studies in a voluminous book entitled "Innere Sekretione," which long remained the bible of endocrinology. As a somewhat lazy student, I was advised to read a shorter English text entitled "*Regulators of Metabolism*" by Noel Patton, professor of physiology at the University of Glasgow. I did this with considerable interest, underlining important facts which I resolved never to forget. I dusted off this book the other night and was surprised to learn that there is an interrelationship between the pituitary and the thyroid of such a nature that when the thyroid is removed the pituitary enlarges and *when the pituitary is removed the thyroid undergoes considerable hypertrophy apparently in order to take over its job.*

Thus, by 1920 there had been sufficient clinical observations and experimental studies to establish convincingly the great importance of the "Secretions internes" which Claude Bernard had postulated in 1857. It was realized, of course, that hormones must exert their effects on metabolic or physiologic processes. Nevertheless, the clinician's interest was attracted mostly by the spectacular morphologic alterations of body build, skin, hair and fingernails which occurred in such syndromes as cretinism, acromegaly, dwarfism, eunuchoidism, Froehlich's syndrome and virilism, which had been so carefully and beautifully described. It was predicted that many new syndromes would be discovered and *formes frustes* or varia-

tions and combinations of the known ones revealed. My teachers, Lewellys F. Barker and Thomas Futcher, and many other astute diagnosticians were keen in this search and examined their patients minutely, applying the relatively few chemical tests where were available.

ENDOCRINOLOGY IN 1935–1940

Let us jump forward 15 or 20 years. In 1935, when Dr. Edwards Park suggested that I organize a pediatric endocrine clinic, I asked him flippantly whether he wanted to turn an honest but poor pediatrician into a charlatan. I showed my ignorance by this remark but I was influenced by some texts of clinical endocrinology and published papers which I read, and lengthy protocols coming from celebrated physicians. Most diagnosticians were seeking and describing more and more subtle morphologic changes and various unimportant signs and symptoms. More chemical laboratory tests were being made; and these were often in error or misinterpreted. One marvelled, but was not convinced, at the ingenuity with which diagnoses were made. Pluriglandular syndromes were being invented in which there was postulated overactivity of glands A and B with hypofunction of glands C, D and E. This tendency was aided and abetted by some pharmaceutical firms which put out preparations which contained varying proportions of testes, pituitary and thyroid with pinches of thymus, pineal and tonsils thrown in for flavoring. To help the less erudite physician in diagnosis, their advertising contained illustrations such as that shown on the following page.

Nevertheless, since 1920 a relatively few but some very important advances in clinical endocrinology had been made. The first parathyroid tumor was removed by Mandl in 1925, a pheochromocytoma by C. H. Mayo in 1927 and an insuloma by Howland in 1929. A few virilizing adrenal tumors had been successfully removed but most patients died.

In July 1921, Banting and Best prepared the first *insulin* and in January 1922 the first diabetic patient, a doctor from Nova Scotia, was treated. This was a great stimulus to attempts to prepare active hormones. In 1927, Abel isolated pure insulin crystals and in the same year Harrington and Barger announced the structure and chemical synthesis of thyroxine. In 1928, Kamm separated the vasopressor or antidiuretic from the oxytocic factor of the posterior pituitary making possible their therapeutic uses.

In 1925, Rogoff and Stewart, in 1927, Hartman *et al.* and in 1931, Swingle and Pfiffner prepared *extracts of the adrenal cortex* which prolonged the survival time of adrenalectomized dogs when they were given abundant NaCl and glucose in addition. Beginning in 1929 these extracts were given to Addisonian patients with some improvement, but the results were far from brilliant. In 1933, Robert Loeb, and shortly afterward George Harrop, treated Addisonian patients with NaCl with some success. Harrop and his group at Hopkins then began to adrenalectomize dogs and were

PRONOUNCED PERSONALITIES

These types are often hereditary but in all cases are associated with peculiar reactions of the glands of internal secretion.

(Reproduced from Stockard. The illustration typifies the kind of advertising that was used early in the marketing of glandular preparations.)

joined shortly by a sandy-haired boy from Buffalo, named Thorn, who collected a famous pack of bassett hounds, favorites of the rabbit hunters in Maryland. As a result of their careful and tedious studies of electrolytes, long before the temperamental flame photometer was known, the role of sodium and chloride in producing the symptoms of Addison's disease was thoroughly elucidated; while the importance of potassium was emphasized by Wilder in 1936.

Between 1923 and 1926, Hanson, a U. S. Army surgeon and Collip in Canada, working independently, prepared active *extracts of the parathyroid glands*. The study of lead poisoning had turned the attention of Joe Aub, tonight's recipient of the Endocrine Society Medal, toward basic investigation of calcium metabolism and bone physiology. The soil was thus prepared for the study of Collip's parathyroid extract by Aub and his brilliant coworkers, Walter Bauer and young Fuller Albright. In the years around 1930 an exchange of residents and fellows between Massachusetts General Hospital and Johns Hopkins brought the Promethean fire from Bostonian Olympus to Baltimore where its phosphorescence inspired Read Ellsworth, Palmer Futcher and our Maryland tennis champion, Johnnie Howard. The dual fires, burning brightly ever since and lighting new conflagrations elsewhere, have led to the description and study of hypo- and hyperparathyroidism, and numerous metabolic bone and renal disorders and the investigation of the effects of parathyroid and other hormones and of vitamin D upon calcium and phosphorus metabolism.

During this same period work was being carried out which seemed less spectacular but was even more important in laying the foundation for the future progress of endocrinology. Anatomists and physiologists, working painstakingly in their laboratories on small animals were devising ingenuous new methods of hypophysectomy and of parabiotic union, which were to reveal the basic relationships of the endocrine system and techniques of bioassay which were to lead to the isolation of pure hormones and to more accurate diagnosis of clinical disorders. A long series of investigations finally clarified the role of the pituitary as the "master gland." As early as 1916, Philip E. Smith studied the effects of hypophysectomy and implanting pituitary tissue in tadpoles and in 1921 Herbert M. Evans was observing the effect of injecting crude pituitary extracts on the growth of rats. By 1926 Smith had perfected the technique for hypophysectomizing rats. In 1926 both Smith and Zondek were carrying out experiments which showed that crude pituitary extracts repaired the gonadal atrophy which resulted from hypophysectomy. In 1928 Ascheim and Zondek discovered that the injection of the urine of pregnant women exerted gonadotropic effect on the ovary of the rat and thereby laid the foundation for the future development of gonadotropic assay. The lists of the pioneers who studied the tropic hormones of the pituitary during the 1930's is too long to enumerate. In the gonadotropic field there were Smith, Fevold, Hisaw,

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van Dyke, Greep, Evans and his coworkers, Riddle, Lyons and others too numerous to mention. At the same time Collip, Pugsley, Evelyn Anderson and others were studying thyrotropic hormone and the Evans group growth hormone. Later these pioneer workers were joined by skillful protein chemists such as Choh Haoh Li, A. White, Cierezsko, Wilhelmi and others. George Sayers and others began to measure the ascorbic acid and cholesterol content of the adrenal as an index of its activity, thereby developing a method for the measurement of ACTH. This phase of work continued for another decade, during which prolactin was isolated by Lyons in 1937 and identified as luteotropin by Astwood in 1941; L. H. was separated from FSH, identified with ICSH, and isolated in 1940; thyrotropin was isolated in 1942; ACTH in 1943; and growth hormone in 1944.

During this era of great discoveries, there occurred a happy union between the endocrinologists and steroid chemists which bore much fruit. The development by Allen and Doisy in 1923 of a method for bioassaying estrogen led to the isolation of estrone by Doisy *et al.* and by Butenandt in 1929, and the synthesis of estradiol by Schwenk and Hildebrant in 1933. Similarly the perfection of the capon-comb androgen assay by Koch and his coworkers enabled his pupil, McGee, to obtain an active alcoholic extract of bull's testes in 1927 and led to the isolation of crystalline testosterone by David, Dingemanse, Freud and Laqueur in 1935 and its synthesis by Butenandt and by Ruszika in the same year. The pioneer studies of the corpus luteum by George W. Corner and the development of a bioassay method by Corner and Allen in 1929 led eventually to the isolation of progesterone by Wintersteiner and Allen in 1934 and its synthesis by Butenandt and by Fernholz within the same year. In the meantime the chemists were studying the steroids of the adrenal cortex, but it was not until 1936 that Mason, Kendall *et al.* isolated Compounds A and B and Reichstein isolated B and named it corticosterone. The following year Steiger and Reichstein synthesized desoxycorticosterone.

The isolation from the urine of steroids shown later to be the degradation products of the sex hormones was likewise of the greatest importance. Pregnanediol was isolated from pregnancy urine by Marrian in 1929 and Butenandt obtained androsterone in 1931. The Zimmerman reaction was discovered in 1935, and in 1938 Callow and coworkers described the method now used with various modifications for the measurement of 17-ketosteroids.

Thus, by the end of the 1930's the basic relations of the endocrine system had been clarified; bioassay and chemical methods which could be applied for studying hormonal secretion in patients with various disorders had been devised; and biologically potent hormones were available to study their metabolic and other actions in the human subject. Tools and methods were now ready to be applied by the clinical investigator.

APPLICATION OF NEW METHODS AND DISCOVERIES TO CLINICAL ENDOCRINOLOGY

During the years between 1935 and 1943, the daily output of urinary gonadotropins and sex hormones were measured and the *norms established* for childhood, adolescence and adult life by Catchpole and Greulich; Nathanson, Towne and Aub, Talbot and coworkers and by the Albright group. Hormonal measurements made by Rakoff and Canterow, by G. W. and O. W. Smith, by Venning and others combined with the careful observations of vaginal and endometrial changes on monkey and man by Carl Hartman, Corner, Hisaw, Earl Engle and others clarified most of the mysteries of the menstrual cycle, the menopause and pregnancy.

The *diagnostic value of gonadotropic assays* was demonstrated in 1942 when Varney, Kenyon and Koch and Albright and his colleagues, showed that they served to differentiate primary gonadal defects from hypogonadism due to pituitary deficiency. Albright then elucidated the syndrome described by Henry Turner, which subsequently became known incorrectly as "ovarian agenesis and stunted growth." Shortly afterward the Albright group and Heller and Nelson applied gonadotropic studies to the diagnosis of primary testicular disorders. During this period Albright was blazing the pathway for more rational diagnosis by showing the importance of studying gonadotropins, urinary 17-ketosteroids and carbohydrate metabolism in suspected pituitary deficiency.

In the early 1940's clinical investigators began to study intensively the *metabolic actions* of the recently discovered hormones which were now available. J. S. L. Browne, Kenyon and his group, McCullagh, and Albright *et al.* and Wilkins and Fleischman studied the protein-anabolic action of testosterone and methyltestosterone and were seeking unsuccessfully new steroids which might be anabolic without virilizing. The metabolic actions of the adrenocortical hormones, aside from their effects on electrolytes, were being studied also. As early as 1927, Cori and Cori had shown that liver glycogen was depleted in adrenalectomized animals, and in 1932 Britton and Silvette had emphasized the role of the adrenal in carbohydrate metabolism. Between 1936 and 1940, C. N. H. Long and his coworkers carried out careful animal experiments elucidating the gluconeogenetic action of the adrenocortical steroids and demonstrated that they increased the deamination of the amino acids. Cushing first described his syndrome in 1932 at a lecture which I attended at Hopkins; but this disorder was thought then to be due to a pituitary disorder. In 1938 Anderson and Haymaker suggested that the symptoms of Cushing's syndrome could all be accounted for by the overproduction of the various adrenocortical hormones. Nevertheless, the manifold symptomatology remained a mystery to most clinicians until it was beautifully elucidated by Albright in his Harvey Lecture of 1942. During the early 1940's there was

also intense interest in the role played by the metabolic effects of both adrenocorticoids and androgen in acute and chronic illness and in various forms of injury and stress.

The Josiah Macy, Jr., Conferences, organized by Dr. Fremont-Smith under the inspiring leadership of Fuller Albright, brought together the various groups of clinical investigators working on these metabolic problems, such as the groups of Albright, John Browne, Bassett, Kenyon, Kochakian, Shorr, Howard and myself, with leaders in steroid chemistry such as Dobriner and Gallagher, Kendall and Mason, and Pincus and Dorfman. These conferences afforded a rare opportunity for interesting and stimulating discussion and interchange of information concerning work in progress. The peak of intellectual activity generally was reached after midnight in Dobie's apartment aided by alcoholic extraction. This conference was concerned also with Ca and P metabolism, the composition of bone, and the effects on bone of parathyroid and other hormones being studied by Albright and Reifenstein, J. E. Howard, Armstrong and others. We were kept informed by Dobriner and Gallagher of their pioneering work in the column fractionation of steroids and the patterns found in carcinoma and various conditions. In these meetings many of us first learned from Dent about paper chromatography and from Stettin about the use of radioisotopes in the study of intermediary metabolism.

During this period, Selye had been studying the effects of stress on the rats' adrenals and in 1946 he elaborated his theories concerning the role of the adrenal cortex in adaptation to stress. The hypothalamic-pituitary-adrenocortical system then assumed its place as the homeostatic regulator of metabolism which enables the organism to meet the needs of stressful influences.

In 1949 a tremendous stimulus to the study of adrenal physiology and of the effects of adrenal hormones upon the peripheral tissues resulted from Hench's discovery that rheumatoid arthritis was favorably affected by cortisone, recently synthesized by Kendall. Within a short time effective preparations of ACTH were made available through the efforts of John L. Mote of the Armour Laboratories. The experimental use of these substances in innumerable diseases and the study of their antiallergic and antiphlogistic actions have shown that the effects of adrenal hormones are not confined to regulating carbohydrate, protein and fat metabolism but have still poorly-understood effects upon collagen, cells and enzyme systems. Dr. Mote deserves great credit for organizing the ACTH Conferences of 1951 and 1952 so that the rapidly accumulating knowledge of scores of investigators could be promptly pooled and evaluated.

The availability of cortisone and related compounds made adequate substitution therapy possible and resulted in therapeutic advances such as the operative cure of Cushing's syndrome and the employment of hypophysectomy for the palliation of cancer. Nothing in medicine is more

spectacular and gratifying than the prevention or control of virilization in female pseudohermaphodites which resulted from the discovery in 1950 that virilizing adrenal hyperplasia can be suppressed by physiologic doses of cortisone.

Simultaneously with clinical investigations during the 1940's and 1950's, chemists had been perfecting methods for the fractionation and isolation of minute amounts of individual steroids in both plasma and urine. These techniques and the use of radioisotopes made possible experiments on the conversion of steroids by tissue slices and homogenates, the perfusion of glands, and the measurement of steroids in blood coming directly from the adrenal or spermatic veins of intact animals. Such experiments carried out by Dorfman, Hechter, Pincus and others of the Worcester group, by Leo Samuels and his pupils, by Dobriner and Gallagher and by Libermann have increased our knowledge concerning the nature of the active hormones secreted by the glands and have established the probable pathways of their biosynthesis, metabolism, degradation and excretion. The preliminary work of Luetscher and the further application of refined chemical and bioassay techniques led to the isolation of aldosterone and the determination of its structure by Simpson, Tate, Wettstein, Neher and Reichstein in 1954.

It should be emphasized that the clinical investigators have made important contributions to basic knowledge by studying the "experiments of nature" which he encounters and which cannot be reproduced in animals. A notable example is virilizing adrenal hyperplasia. The suggestions of Bartter, of Jailer and of Kelley that this disorder might be due to a defect in the normal synthesis of hydrocortisone resulting in the excessive production of androgenic steroids gave a great impetus to the study of steroidal patterns before and after treatment with cortisone. The studies of Dorfman and of Bongiovanni and Eberlein have demonstrated that there are two different defects in the hydroxylation of progesterone, one of which accounts for the coexistence of hypertension in certain cases of the virilizing syndrome. Similarly the discovery and study of goitrous cretinism in regions where there is no deficiency of iodine has led to renewed interest in the pathway of synthesis of thyroid hormones and the demonstration by Stanbury, McGirr and Hutchinson, Pitt-Rivers and others that hypothyroidism can be caused by enzyme defects. These findings in virilizing adrenal hyperplasia and in goitrous cretinism lead to the new concept that some endocrine disorders may be due to inborn, genetically-determined defects of enzymes necessary for normal hormonal synthesis.

Time does not permit an enumeration of the many advances of endocrinology during the last few years. I can only mention the culmination of du Vigneaud's work of 30 years in the determination of the exact structure and in the synthesis of vasopressin and oxytocin. Attention has focused again on the neurohumoral secretions of the hypothalamus and on attempts

to localize in this tiny structure the exact areas of specific functions. The works of experimental embryologists such as Jost and Wells who studied the effects of castration in very young embryos, and of cytologists such as Barr, who studied sex chromatin patterns, have combined with the observations of endocrinologists and clinicians in helping elucidate some of the mysteries of hermaphroditism and gonadal dysgenesis.

Thus, the embryologist, anatomist and cytologist; the physiologist and clever animal experimenter; the enzymologist and geneticist; the chemist skilled in steroidal methodology and synthesis, in protein structure and fractionation or working on the structure of iodinated compounds; the clinical diagnostician or investigator interested in electrolyte or carbohydrate metabolism, in bone disorders and calcium metabolism, or in renal physiology, have all combined to advance endocrinology. I know of no scientific organization other than our Endocrine Society, which combines in common interests so many experts with totally different skills and disciplines as I see before me tonight. Your work has not only elucidated the endocrine disorders and facilitated their diagnosis and treatment but has contributed greatly to the advancement of all physiology and biochemistry and the understanding and treatment of disease.